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A CONTRIBUTION TO THE DYNAMICS OF TOXICITY AND THE THEORY OF DISINFECTION

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I. INTRODUCTION

It has long been known that the effects of a toxic agent acting upon bacterial or other forms of protoplasm become manifest only after the lapse of a shorter or longer period of time. Some thirty years ago Abbott (1891), studying the disinfection of *Staphylococcus pyogenes-aureus* by mercuric chloride, concluded that the explanation for the time curve must lie with differences in the resistance of individual cells in a bacterial population to the lethal agent. Since then numerous workers have studied the course of disinfection processes by various quantitative methods with the hope that such studies might cast some light upon the nature of the reaction between toxic agent and cell and lead to an explanation of the shape of the time curve of the process. Admittedly, the physical and chemical systems involved in the structure of protoplasm are extremely complex and the reactions between a toxic agent and the constituents of these systems may be equally complex. None the less, it has been the hope of many investigators that some simple physical or chemical reaction between disinfectant and protoplasm would be found to suffice as an explanation for the dynamics of the lethal process. And some have considered that these hopes have been attained.

In recent years there have been published several reviews of the extensive literature on the theory of disinfection dynamics. Hence it is not our intention to present more than a few comments on earlier papers and only such as are especially pertinent to the purposes of the present study. Critical discussions may be

consulted in the papers of Brooks (1918–1919), Eisenberg (1918–1919), Cohen (1922), Reichenbach (1922), Falk (1923) and Fulmer and Buchanan (1923).

In their classic contribution, Krönig and Paul (1897) reported, the results of careful studies on disinfection by mercuric chloride and succeeded in demonstrating conclusively that the process of disinfection is a gradual and not an instantaneous one, and that it follows an orderly sequence. From their data, Ikéda (1897) found that the curve prepared by plotting the logarithms of surviving organisms against elapsed time is a straight line and that the rate of the disinfection process is in agreement with the rate calculated from the equation of an unimolecular reaction. In a like manner, Miss Chick (1908, 1910, 1912, 1913) was able to calculate a similar, essential concordance with the logarithmic rate from the data of Clark and Gage on the disinfectant action of sunlight and from her own experiments with silver and mercury salts, phenol, heat, blood serum, etc. Numerous other workers (Madsen and Nyman, Cohen, etc.) have obtained essentially the same results. However, nearly all these workers have observed occasional deviations in the logarithmic curve from a straight line, such deviations being especially common in the earliest and latest periods of the disinfection process. Bellei, Loeb and Northrop, Eijkman, Hewlett, Reichel, Reichenbach, Brooks, Peters and others have raised objections to an interpretation of these observations in terms of a simple unimolecular reaction. Indeed, some workers have contended "that such resemblances as have been found between such curves and unimolecular reaction or logarithmic curves are superficial and fortuitous. Any method therefore of evaluating disinfecting power based upon such a concept must prove misleading."[1] With these few introductory remarks for orientation we may proceed to an examination of some new experimental data and then return to a more careful reconsideration of the theoretical aspects of the problem.

The experiments here reported were originally designed as part of an extensive study to determine the effects of certain inorganic

[1] Fulmer and Buchanan, 1923, 88.

salts upon bacterial viability and to cast some light upon the dynamics of the processes of cellular death and of disinfection. It is our plan to present for analysis here only a few of hundreds of similar experiments on the rates of dying of *Bacterium coli* in salt solutions, choosing for our present purposes the data from typical findings upon the mortality in aqueous sodium and calcium chloride solutions of various concentrations and in distilled water at various pH values.

II. EXPERIMENTAL METHODS

Our experiments were conducted with a single strain of *Bacterium coli* which was isolated from a polluted stream near New Haven in the autumn of 1916. It was strain 38 of the original collection of Winslow and Cohen (1918). This strain of *Bact. coli* maintains itself in distilled water at a favorable pH value without material decrease in numbers for a period of nearly twenty-four hours, actual increases being not uncommon during the first few hours. Occasionally, however, a particular suspension will show a marked decrease due to some cause which we have not yet determined. The details of technique used in these studies have been reported in earlier papers (Winslow and Falk, 1923a, 1923b) and need not be repeated.

III. GENERAL RESULTS

In table 1 we cite the data taken from a typical experiment, 37-C, to indicate certain characteristics of the viability curve of *Bacterium coli* in a comparatively dilute solution of $CaCl_2$ at 37°C. The velocity constants of the mortality processes have been calculated from the well-known equation for an unimolecular chemical reaction:[2]

$$0.434\, K_1 = \frac{1}{t} \log \frac{a}{a - x} \tag{1}$$

where a = the original number of viable bacteria

$a\text{-}x$ = the number of viable bacteria after time t

[2] Throughout this paper we have utilized the equations for chemical reactions which are direct and isolated, on the assumption that the changes which finally lead to loss of viability are irreversible. Cf. the theoretical treatment of the dynamics of reactions in homogeneous systems by Rice (1924).

t = time in hours
K_1 = velocity constant
log = logarithms to the base 10

Here we observe that the average value of K_1 is 0.232 and that the individual values of the velocity constant range between 0.213 and 0.256, with a probable error (P.E.) of ±0.011, or approximately 5 per cent.[3] For a biological experiment in which the errors of method involved in various of the procedures used in the technique of bacterial quantitation are appreciable, such a constancy of K_1 as is indicated by a P.E. of less than 5 per cent is

TABLE 1

The viability of Bact. coli in 0.145 M $CaCl_2$ solution

Experiment 37-C

INCUBATION PERIOD—t-HOURS	NUMBER OF VIABLE BACTERIA	LOGARITHM OF VIABLE BACTERIA	VELOCITY CONSTANT—K_1
0	15,200,000	7.182	
1.83	9,500,000	6.978	0 256
5 33	4,700,000	6.672	0.220
7.83	2,390,000	6.378	0.237
21.33	163,000	5.212	0.213

Average K_1 = 0.232
P. E. = ±0.011

striking. From the uniformity of the values of K_1 it follows that if the course of bacterial mortality is illustrated by a graph of logarithms of surviving organisms plotted against time the curve will approximate a straight line. That this is the case is evident from inspection of figure 1. There can be no doubt that such an experiment as this furnishes a general confirmation of the findings of Krönig and Paul, Madsen and Nyman, Chick, Cohen and others who have described the concordance between a bacterial mortality curve and the logarithmic straight line of the unimolecular mass action equation. It is also significant to observe that the mortality rate was comparatively low, approximately two-thirds of the bacteria having survived for a period of nearly two

[3] The probable error of the average was calculated on the basis: P. E. = 0.6745 $\Sigma d^2 n$ where Σd^2 = the sum of the squares of the deviations from the average; and n = the number of values averaged.

hours. Experiments of this kind were specifically designed to meet the issue which has been raised by Loeb and Northrop (1917) and others that in the initial stages of a disinfection process, there is a lag period and that the logarithmic law is not followed if the rate of the process is measured in the early stages of the reaction.

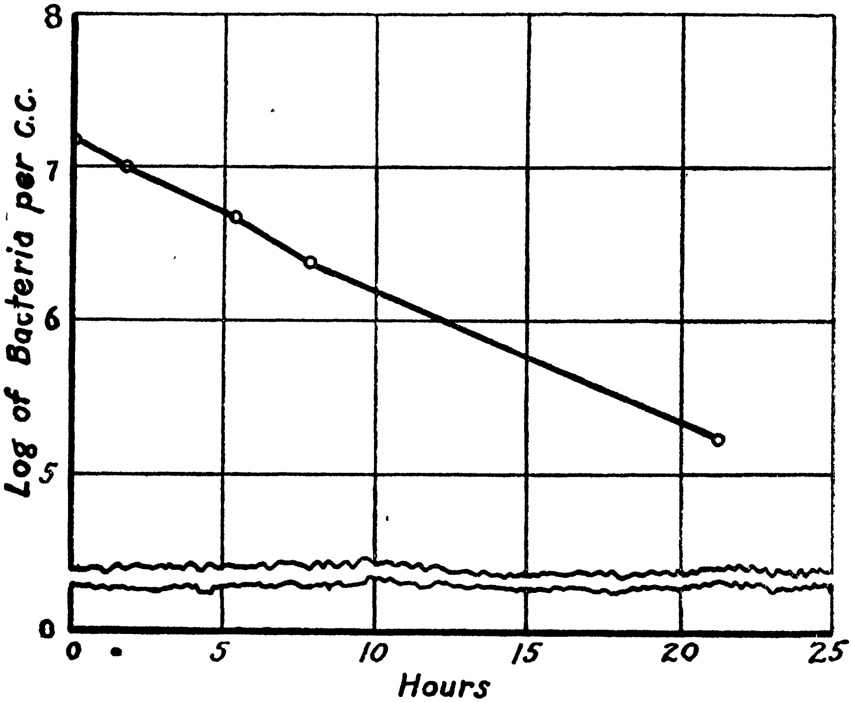

FIG. 1. BACT. COLI IN 0.145 M $CaCl_2$ SOLUTION AT 37°C.

In table 2 we have presented the results of typical experiments on the course of mortality in a bacterial population exposed to various concentrations of calcium chloride. (All of these tests were made at the same time, under similar conditions and with the same technique. Solution C and the data in column (4) are identical with experiment 37-C of table 1.)

From an examination of column (2) of the table it appears that in the presence of a very low concentration of calcium chloride

(0.00145 M) there was a slight multiplication of the bacteria.[4] It also appears that there was no multiplication of the bacteria in the first time interval (0 to 1.83 hours), an appreciable multiplication in the second interval (1.83 to 5.33 hours) a maximal multiplication in the third interval (5.33 to 7.83 hours) and a lesser multiplication in the final period (7.83 to 21.33 hours). From column (3) it appears that there was an even more marked multiplication of the bacteria in the presence of a ten-fold higher

TABLE 2

The viability of Bact. coli in $CaCl_2$ solutions of various concentrations at 37°C.

INCUBATION PERIOD—t-HOURS (1)	VELOCITY CONSTANTS (K_1) IN:				
	A 0.00145M (2)	B 0.0145M (3)	C 0.145M (4)	D 0.725M (5)	E 1.450M (6)
0					
1.83	0	0.101	0.256	1.93	3.36
5.33	−0.178*	−0.384	0.220	1.07	1.93
7.83	−0.203	−0.636	0.237	1.14	∞†
21.33	−0.036	−0.037	0.213	0.766	....
Average.. ..	−0.104	−0.0239	0.232	1.23	2.65‡
P. E..	±0.059	±0.199	±0.011	±0.43	

* A minus sign (−) before a value of K_1 indicates that the bacteria had *increased* in numbers.

† An infinity value (∞) indicates that the reaction went to completion in the time interval (i.e. no viable bacteria remaining). Such a value, calculated by setting $(a-x) = 0$ is inconsistent with the mass action law. Calculated values of K_1 will range from $\left(\frac{2.3}{t} \log a\right)$ to (∞) according as the bacterial count is taken between *1* and *o* per cc.

‡ Average calculated from the two finite values.

concentration of the salt (0.0145M), the rate of growth again attaining a maximal value in the third interval.[5] Columns (4),

[4] It is recognized that the application of the unimolecular equation to the tests in which there are observed *increases* in the number of viable bacteria is not strictly sound. It is carried out for the sake of uniformity of procedure and in order to provide in the $(-K_1)$ values a convenient measure of the rate of multiplication.

[5] The stimulating effect at this salt concentration was unusual. As will be shown later on a concentration of 0.01 M $CaCl_2$ generally proves slightly toxic.

(5) and (6) representing data from viability experiments with solutions of higher salt concentrations (0.145M, 0.725M and 1.450M) show no multiplication of the bacteria, but, instead, progressively higher mortality rates.

It would appear, then, from these data that in very dilute aqueous solutions $CaCl_2$ stimulates *Bact. coli* to multiply and that in higher concentrations it is highly toxic. Now, it is especially significant to observe that in one of the three toxic solutions (column (4)) the course of mortality follows that of an unimolecular reaction, witness the sensible constancy of the values of K_1. If only this particular concentration of $CaCl_2$ had been studied and had been presented alone as in table 1, it would have implied a confirmation of the unimolecular disinfection theory. However, examination of the last two columns of table 2 brings out clearly that with more toxic concentrations of $CaCl_2$ the values of K_1 are not even approximately constant. They show, instead, values which decline steadily for successive intervals of time. If the constancy of K_1 in solution C means that the reaction between $CaCl_2$ and *Bact. coli* is essentially unimolecular,[6] it becomes impossible to reconcile the data in column (4) with those in columns (5) and (6) without making additional assumptions.

If the course of a disinfection process be such as to give the logarithmic straight line of an unimolecular reaction and if it be assumed that the fundamental reaction is unimolecular, we might consider that of the reacting agents (1) disinfectant, and (2) living cell, one or the other is present in excess and that its concentration is not changing significantly during the course of the reaction. Inasmuch as the constant K_1 obviously changes with a change in salt concentration it must be assumed that the reacting substances of the bacterial cell (whether those cells are dead or alive) must always be present in excess if anything approaching the monomolecular curve is to be attained.

[6] Assuming that one of the reacting agents is present in excess and that hence its concentration remains essentially constant. In such a case, the reaction may really be of a higher order, but it will *appear* to be of the first order.

IV. DEVIATIONS FROM THE LOGARITHMIC CURVE

We may now proceed to consider the deviations from a regular logarithmic curve which are manifest on a closer analysis of the results of such experiments as are under discussion. Almost all the observers who have studied this problem report such deviations. At the beginning of the observation period marked and more or less irregular mortalities are manifest and during the later course of an experiment there is a rather regular tendency for the rate of dying to become progressively less. Even in the work of Chick this tendency is clearly manifest.

TABLE 3

Viability of Bact. coli in distilled water

INCUBATION PERIOD—*t*-HOURS	VELOCITY CONSTANTS (K_1) AT pH = :						
	4 0	5.0	6 0	6 5	7 0	7.5	8 0
0							
1	0 14	0.14	0.18	0.09	0.39	0.25	0.23
3	0.31	0.11	0.10	0.14	0.21	0.47	0.21
6	0.54	0.12	0.07	0.20	0.24	0.42	0.35
9	0.51	0.04	0.02	0.29	0.17	0.34	0.23
24	(*)	0.12	0.01	0.16	0.06	0.15	0.10
Average K_1..............	0.37	0.11	0.08	0.18	0.21	0.33	0.23
Number of experiments...	2	2	4	2	10	2	4

* See table 2, footnote †, for explanation.

In all viability studies there are marked irregularities, such as we are accustomed to find in biological investigations,—which make the elucidation of the more subtle influences at work exceedingly difficult. The only way to overcome such difficulties is by the use of averages obtained from the results of large numbers of experiments, and we have therefore attempted to throw some light upon the problem in hand by working out such averages for a considerable number of experiments. These data are presented herewith in tables 3 to 5 and figures 2 and 3. In these tables the values for K_1 have been expressed only in two decimal places since

the chance errors are such as to rob the third decimal of any practical significance.[7]

From tables 3 to 5 it is obvious that there is a rough general approximation to a constancy of K_1 with a given concentration of a given toxic substance. In distilled water solutions near

TABLE 4

Average reaction velocities of mortality of Bact. coli in various concentrations of $CaCl_2$, and after various periods of exposure

CONCENTRATION $CaCl_2$ NORMALITY	NUMBER OF TESTS	HOURS					
		1	3	6	9	24	Average
0.001–0.002	1–4	−0.18	0.04	−0.10	−0.05	−0.02	−0.06
0.01–0.03	28–31	0.32	0.10	0.09	0.05	0.01	0.114
0.07–0 14	20–24	0.16	0.18	0.17	0.12	0.09	0.144
0.4	2–11	0.84	0.80	0.73	0.60	0.48	0.690
0.7	3–15	1.30	1.34	1.01	1.17	0.82	1.128

TABLE 5

Average reaction velocities (K_1) of mortality of Bact. coli in various concentrations of NaCl and after various periods of exposure

CONCENTRATION NaCl NORMALITY	NUMBER OF TESTS	HOURS					
		1	3	6	9	24	Average
0.01–0.03	7–8	0.00	0.00	−0.03	−0.02	−0.02	−0.002
0.07–0.14	9–11	0.11	0.09	0.10	0.09	0 08	0.094
0 4	6–8	−0.05	0.14	0.17	0.24	0.03	0 106
0.7	19–20	0.04	0.24	0.24	0.22	0.23	0.194
0.9–1.0	14–16	0.12	0.34	0.29	0.20	0.09	0.201
1.4	5–15	0.44	0.71	0.81	1.22	0.48	0.732

neutrality and in dilute salt solutions K_1 is very small, that is the rate of bacterial mortality is low. A very dilute solution of either NaCl or $CaCl_2$ is definitely favorable to survival, causing in general an actual increase in numbers ($-K_1$). With an acid concentration equivalent to pH 4.0 or an alkaline concentration equivalent to pH 7.5 the mortality constant increases as it does

[7] The averages have been obtained arithmetically,—a procedure not perhaps justified on mathematical grounds, since we are dealing with logarithmic terms, but indicating general trends with an accuracy which has been found by calculation to be sufficient for the present discussion.

with NaCl concentrations of 1.0 M or over and with a $CaCl_2$ concentration of 0.4 M and over.

Closer analysis of the figures shows, however, rather definite deviations from a constant value of K_1. Even with the distilled water data in table 3 it is apparent that the K_1 values are generally

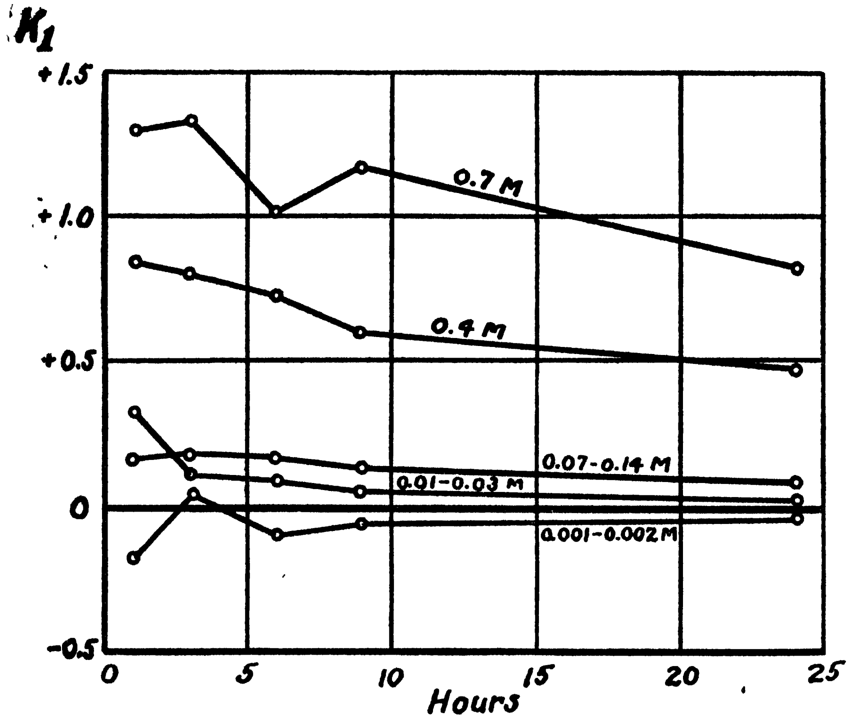

FIG. 2. BACT. COLI IN $CaCl_2$ SOLUTIONS OF VARIOUS MOLARITIES

lower after twenty-four hours than at earlier periods. This fact is brought out much more clearly by the averages based on more abundant data in tables 4 and 5 and figures 2 and 3.

In the $CaCl_2$ solutions (see fig. 2) every one of the five curves shows an almost steady decrease in K_1 with the progress of the experiment. In the NaCl solutions (fig. 3) there is apparently an increase in K_1 during the first few hours with a subsequent decrease. In the 1.4 M concentration the variation is so great as to eliminate almost all semblance to the curve of a monomolecu-

lar reaction. This may in part be due to the fact that while the averages for one, three and six hours are based on 15 to 16 observations, those for nine and twenty-four hours are based only on 5 observations. Within the series of 5 experiments carried through the whole twenty-four hours the same relation is, however, manifest; the average K_1 values for these five experiments

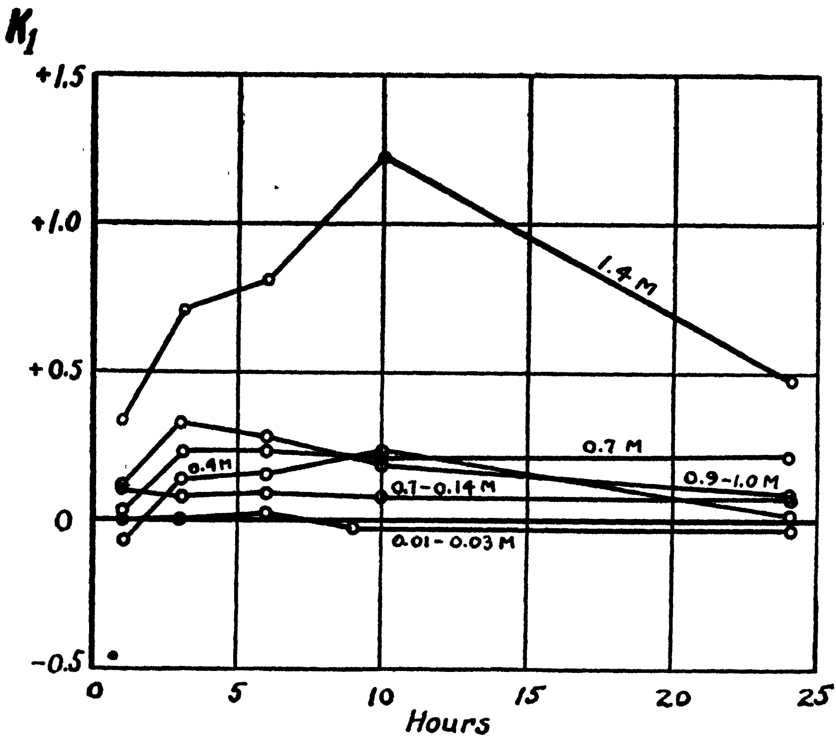

FIG. 3. BACT. COLI IN $NaCl_2$ SOLUTIONS OF VARIOUS MOLARITIES

being as follows: 1 hour, 0.37; 3 hours, 0.57; 6 hours, 0.88; 9 hours, 1.22; 24 hours, 0.48. It appears to us probable that there is a real increase in K_1 values during the first few hours of exposure to NaCl solutions; and there is certainly a progressive decrease in K_1 during the later hours in most of the solutions studied.

V. CRITICISMS OF THE VIEW THAT MORTALITY FOLLOWS A LOGARITHMIC CURVE

Most of the investigators who have studied the dynamics of disinfection,—Krönig and Paul (1897), Ikéda (1897), Madsen and Nyman (1907), Chick (1908) and Cohen (1922),—have been chiefly impressed with the broad general similarity between the curve of viability and that of a monomolecular reaction. Their general conclusions have been essentially as follows:

a. Disinfection is a gradual and not an instantaneous process.

b. Under the prescribed conditions, it generally follows an orderly sequence.

c. The course of the process under these conditions is generally logarithmic and is described by the equation of an unimolecular reaction.

Among these workers there have been differences of opinion on the interpretation of the findings but not on the nature of the findings themselves. Three other investigators on the other hand have specifically questioned the validity of conclusions *b* or *c* (or both) listed above.

First of all, Loeb and Northrop found in their work with Drosophila (fruit fly) that the mortality curve at higher temperatures simulates a probability distribution curve and is not of the nature of a mass action logarithmic curve. As has been pointed out in another connection, these authors in discussing the work of Miss Chick, overlooked the fact

> that Miss Chick's unimolecular reaction curve is not an assumption (as they state) but a deduction, and that there is no inherent reason why there should be an essential similarity between the equation which describes the rate of mortality of bacteria subjected to a large excess of toxic substances and the rate of mortality of fruit flies whose span of life—according to Loeb and Northrop—is probably limited by the production within the animal, in the course of its own metabolism, "of a substance leading to old age and natural death or by the destruction of a substance or substances which normally prevent old age and natural death" (Falk, 1923, pp. 95–96).

It appears, also, that there may be but scant validity in the comparison of mortality data for *Drosophila melanogaster*—an

animal form which shows bisexuality and differentiation between soma and germ plasm, and bacteria—plant forms in which neither sexual nor somatic specilizations are demonstrable.

The second of these critics of the monomolecular reaction hypothesis, Brooks (1918) showed that if erythrocytes are suspended in an indifferent medium and are subjected to a hemolytic agent (radiation from a mercury vapor arc or specific hemolytic antibody) the curve of liberated hemoglobin plotted against sampling time gives not the curve of a mass action reaction but of an asymmetrical sigmoid, i.e., showing an initially slow rate of hemolysis, an increasing rate to a maximum, a gradual retardation and the attainment of an equilibrium. Without going into the details of his arguments, we may indicate that Brooks interprets this finding by postulating the existence of differences in resistance of the cells to the hemolytic agent. Brooks further distinguishes between the resistances of the cells to the hemolytic agent which determine the "course of the process" (i.e., the time curve) and the "fundamental reaction" (i.e., "the physicochemical processes in the protoplasm") and treats each as an independent variable. Hence, he concludes that the time curve cannot directly indicate the nature or order of the fundamental reaction.

Finally, Fulmer and Buchanan (1923) have reported the results of a series of experiments on the toxicity of phenol and phenol and alcohol for yeast cells. Their measurements of toxicity were made by staining periodic aliquot portions taken from a yeast cell suspension exposed to the toxic agent. They say:

> It has been shown that all cells which take up the stain are dead, that is, they no longer can reproduce. However, it was found that the ability to reproduce was lost *before* the cell acquired the ability to stain. In other words, cells which stain are dead, but a dead cell does not of necessity immediately acquire the ability to stain. The two phenomena are closely related, so that cell staining is a satisfactory criterion of toxicity (p. 79).

Of twenty-seven sets of experiments which they cite, they find that in four there was no period with a maximal rate of dying,

in nineteen there was one and that in four there were two well marked maximal periods. They conclude, therefore, that their time curves do not in general prove to be logarithmic; that such resemblances as have been found between such time curves and unimolecular or logarithmic curves are superficial and fortuitous; and that variations in resistance of individual cells and the distribution of such variations must be regarded as of fundamental importance in accounting for rates of death of microörganisms.

The finding of Fulmer and Buchanan that the time curves of their yeast disinfections are not even in a general way logarithmic, and their conclusion that such resemblances as have been found are "superficial and fortuitous" demands critical evaluation.

It is recognized that staining with dilute methylene blue may be—as Fulmer and Buchanan state—an adequate test for the mortality of the cell. It must also be recognized, however, that such a technique may serve as an adequate criterion of completed toxicity, i.e., of lethality, without providing a criterion for measurement of *rate* of lethality, unless it be first demonstrated that the time interval which must elapse after the death of a cell before it displays avidity for the stain is (1) constant for all cells in a test suspension, (2) is invariate with different toxic agents, and (3) is sensibly small by comparison with the time requisite for the effective action of the toxic agent. The reasons for conditions (1) and (2) are self evident. Condition (3) arises out of the consideration that—assuming fulfillment of (1) and (2)—the rate curve would show anomalous inflections, especially at the beginning and end, if the time for development of avidity for the stain is appreciable by comparison with the time necessary for the disinfectant to exert toxicity. Thus, if the first interval of time on a lethality rate curve were equal to the time which elapses between the establishment of the status which is measured by loss of reproductive capacity and the development of avidity for the dye, the rate of dying in this first period—as measured by the staining reaction—would be zero. In the next interval of time it would be something greater than zero,—in the direction of a positive value. In successive intervals the rate would increase and approximate the true rate of dying. As the end of the dis-

infection was approached, the rate would in a similar manner decline from the true value for the rate of mortality. Fulmer and Buchanan picture curves of just the kind which could be deduced from this analysis. (A precise, mathematical treatment of consecutive reactions is presented by Mellor, 1904, pp. 113 ff. and is reconsidered in its biological applications by Osterhout, 1922.) It is also pertinent to note that if initial and final lag deviations from a logarithmic straight line are attributable to the catenary nature of the fundamental reactions involved in the development of dye avidity by yeast cells, there should be no inflection in the direction of terminally accelerated rates. Nor are any clearly marked inflections of this kind observed by Fulmer and Buchanan.

VI. CONCLUSIONS IN REGARD TO THE REGULARITY OF DISINFECTION PROCESSES

It appears from a general review of the subject that while Fulmer and Buchanan find no regularity in the disinfection process as measured by the stainability of yeast cells, all of the other workers with microbic viability report a general correspondence with the logarithmic curve but with more or less distinct deviations toward the beginning and the end of the process. Cohen's curves are, on the whole, the most regular with which we are familiar. Even in his studies, however, many of the curves for mortality at 30° show a lessened rate of reduction toward the end. Chick's figures reveal much greater deviations from the monomolecular straight line, although she is inclined to minimize them in her discussion. Our own studies, as pointed out above, show a marked and definite tendency toward a decreasing rate for K_1 as the process of disinfection continues.

It seems, in general, that while the rate of disinfection follows a logarithmic curve it does not, as a rule, follow it strictly; and the degree of deviation from a monomolecular reaction varies with the particular type of disinfection which is going on. The death of bacteria in water at low temperature follows the monomolecular curve very closely (Cohen); the destruction of bacteria by standard disinfectants (Chick) deviates slightly from it; the mortality

in presence of NaCl and $CaCl_2$ (our own data) shows greater differences; the mortality of Drosophila (Loeb and Northrop) and the hemolysis of red blood cells (Brooks) differ still more; while the stainability of yeast cells after exposure to phenol and alcohol exhibits very little correspondence with the monomolecular law. It appears to us that this is exactly what might be expected, since it seems inherently very improbable that all these various processes destructive of protoplasm should be of the same simple chemical nature.

VII. POSSIBLE EXPLANATIONS OF DEVIATIONS FROM THE LOGARITHMIC CURVE

Where deviations from the logarithmic curve are actually present, we are by no means convinced that the assumption of variability on the part of the reacting cells is a necessary or an altogether satisfactory one. It is significant to recall that "resistance" of a cell to a lytic or to a lethal agent is not an independently *measurable* factor. "Resistance" is known only as a function of time. Thus, a cell is "more resistant" to a lytic or a disinfectant agent if a longer period—and is "less resistant" if a shorter period—of time must elapse before it is lysed or killed. We are not aware that "resistance" is known in any other terms or is measurable in any other units. It represents, therefore, a state whose existence is *assumed* but not *proved.*

In the field of chemical phenomena it is not clear why molecules, atoms or ions which eventually take part in a reaction do not all react to the completion or equilibrium of the reaction in a single unit of time.[8] In the terms of the kinetic theory, it was customary to consider that the interactions were determined by the occurrences of collisions or bombardments and that these were determined by chance (that is, by the operation of a large number of factors), and by the nature and the concentrations of each kind of substance. On this ground, however, it became difficult to account for the fact that the temperature coefficients of most chemical reactions are much greater than postulates of the ki-

[8] Cf. Rice (1924, p. 898, et seq.).

netic theory would warrant. In terms of the quantum theory, as Cohen (1923) has indicated, the explanation for the time curve would lie, presumably, with the chance distribution of variable energy quotas in the molecules of a substance. For the time curve of a disinfection process, Chick (1908) was unable to conceive the existence of such differences in the "resistance" of the cells to the disinfectant as could account for its shape. She considered instead, from analogy with the dynamics of heat coagulation of proteins, which closely resemble the destruction of bacteria by hot water, that "an explanation has been sought in temporary changes in the energy of the molecules, as a consequence of which all molecules do not possess the same sensibility to attack at the same moment. Some such property is therefore attributed to the molecules (or aggregates of molecules) of the constituent protein of the bacteria, whereby at a given moment only a certain proportion is liable to attack; the amount being dependent upon the concentration at the moment of unaltered protein, in other words the number of bacteria surviving in unit volume."[9] It is at once evident that these views are essentially the same as those deduced from general chemical or thermodynamical considerations. From this standpoint while the curve for an array of bacteria of uniform chemical composition should show a monomolecular curve of mortality, a population of variable composition (resistance) might be expected to yield a curve made up of several different logarithmic curves, each component curve corresponding to a different degree of resistance.

Some years ago, we pointed out (Winslow and Falk, 1920) that the postulation of such a specific resistance factor was unnecessary and that it needlessly complicated the concepts of disinfection dynamics. We also pointed out that the results obtained from quantitative experiments can be explained if it be assumed that the death of a cell be due not to the results of a single

[9] Similarly, Arrhenius (1915) had clearly recognized the discrepancy between a postulation of different resistances in a bacterial population and the observation of a logarithmic course in disinfection. He concluded that "the different lifetime of the different bacteria does not, therefore, depend in a sensible degree on their different ability to resist the destructive action of the poison" (pp. 78–79).

reaction but to a chain of reactions (i.e., conceiving the fundamental reaction of Brooks as a catenary reaction). Brooks himself considered that "the fundamental reaction may be either a simple process, or the expression of a complex series of changes whose rate is at all times governed by that of the slowest of the series" (p. 79).

In the field of chemical dynamics it is known that a reaction between reagents may be of one order with one set of concentrations and of another with a second. Thus it appears that the reaction between potassium ferricyanide and potassium iodide works out to be unimolecular with respect to the former component, whereas at other concentrations it is shown to be bimolecular (Mellor, 1904, p. 64; also, Rice, 1924, p. 883). In table 6 we cite the results of our Experiment 47-B on the course of mortality for *Bact. coli* in 0.145 $CaCl_2$ solution, showing the velocity constants (K_1, K_2, K_3, and K_4) which were calculated from the following equations for uni-, bi-, tri-, and quadri-molecular reactions:[10]

$$K_1 = \frac{2.3}{t} \log \frac{a}{a - x} \tag{2}$$

$$K_2 = \frac{1}{t} \frac{x}{a(a - x)} \tag{3}$$

$$K_3 = \frac{1}{t} \frac{x(2a - x)}{2a^2 (a - x)^2} \tag{4}$$

$$K_4 = \frac{1}{t} \frac{1}{3} \left(\frac{1}{(a - x)^3} - \frac{1}{a^3} \right) \tag{5}$$

From column (2) it appears at once that the disinfection did not follow the logarithmic course illustrated in a similar experiment cited in table 1 (the same concentration of $CaCl_2$ was used in the two cases) as is evident from the inconstancy of K_1. The steadily decreasing values of K_1 indicate that the reaction between disinfectant and viable cell was proceeding at a rate higher than that

[10] It should be clearly understood that these equations represent only first approximations for the present purposes. The actual concentrations of the two, three or four reacting molecules cannot be determined and are therefore taken as equal merely for the sake of making these first approximations.

indicated by the so-called logarithmic law. Further, if the first and last two values of K_2 (column 3) be disregarded, the reaction may be considered to have proceeded in accordance with the course of a bimolecular chemical reaction. The approximately constant values of K_3 in column (4) suggest, however, that after the first three or six hours, the reaction proceeded in the manner of a chemical reaction of the *third* order. These observations are confirmed by the progressively increasing values of K_4, listed in the last column, which indicate that the course of the disinfection rate was lower than that postulated from the equation of a *fourth* order reaction. In brief, then, the data of table 6 indicate that when the disinfection of *Bact. coli* by 0.145 M $CaCl_2$ does not fol-

TABLE 6

The viability of Bact. coli in 0.145 M $CaCl_2$ solution at 37°C.

INCUBATION PERIOD—*t*-HOURS (1)	VELOCITY CONSTANTS			
	K_1 (2)	K_2 ($\times 10^8$) (3)	K_3 ($\times 10^{16}$) (4)	K_4 ($\times 10^{24}$) (5)
1	1.56	2.14	3.39	6.50
3	0.65	1.16	2.59	7.01
6	0.46	1 39	6.48	39 3
9	0 34	1 30	8.23	68.9
12	0 27	1.17	8.50	83.9
24	0 15	0.85	8.92	127.0
125	0 034	0.36	8.00	236.0

low the logarithmic course of a unimolecular reaction in a particular experiment, it may be following the course of a reaction of a higher order through a considerable period of time (i.e., *second* order, 1 to 12 hours; *third* order, 9 to 125 hours in table 6). Further, we may even consider that by the method of best fit of the equations the course of disinfection in experiment 47-B was chiefly that of a third order chemical reaction with a lag in the early stages which approximated the course of a second order reaction.

We come now to the consideration of a phase of disinfection theory which is suggested by our experimental findings and which hitherto appears to have been neglected. More than thirty

years ago, Richet (1892) pointed out that for each of a considerable series of salts whose influences on lactic fermentation of milk whey were studied it is possible to demonstrate that there are concentrations which are:

a. Indifferent
b. Stimulating
c. Inhibitive
d. Toxic

His series included salts of Na, K, Li, Mg, Ca, Sr, Ba, Fe, Pb, Zn, U, Al, Cu, Hg, Au, Pt, Cd, Co, and Ni. His findings, especially with respect to the existence of the stimulating concentrations, are the more noteworthy because of the inclusion in his series of salts with such highly toxic cations as those of cadmium, cobalt, nickel, copper, gold, platinum and mercury. Recently, Dr. Hotchkiss (1923), unaware of Richet's work, came to essentially the same conclusions from more carefully conducted experiments on the stimulating and inhibiting influences of a series of salts upon the growth of *Bact. coli.*[11] She was able to find for 15 of 23 chlorides studied a concentration which stimulated growth—as indicated by the production of a turbidity greater than that in the control cultures to which no salt had been added. The stimulating salts in her series included not only chlorides of K, Na, NH_3, Li, Sr, Mg, Ca and Ba, but also of such toxic metals as Ti, Sn, Ni, Pb, Co and Hg; and she was persuaded that stimulating concentrations for the other eight salts could have been established by more exhaustive study. Madsen and his associates have recenty reported[12] similar stimulating effects of minute amounts of Cu and Mn upon the growth of the tubercle bacillus.

Accepting these conclusions as indicative of a general biological principle,[13] it is of considerable interest to reëxamine from this point of view the course of mortality of *Bact coli* in the presence of various concentrations of calcium chloride. In a more detailed report (Winslow and Falk, 1923a) and in tables 4 and 5

[11] The same strain as that used in our studies.
[12] Meeting, Laboratory Section, A.P.H.A., Detroit, November, 1924.
[13] Further evidence is cited at length in a review by Falk (1923).

we have indicated that either sodium chloride or calcium chloride, in suitable concentrations, may be stimulating as well as toxic to *Bact. coli* suspended in non-nutrient menstrua. If from our data average velocity constants of mortality (K_1) are calculated (assuming, tentatively, the applicability of the unimolecular reaction equation), we obtain (see last columns of tables 4 and 5) a series of values which pass from negative to positive values and which give a reasonably smooth curve for both salts (fig. 4). We are not aware that any chemical theory of disinfection which has previously been presented is in harmony with the fact that these curves are approximately continuous, above and below the

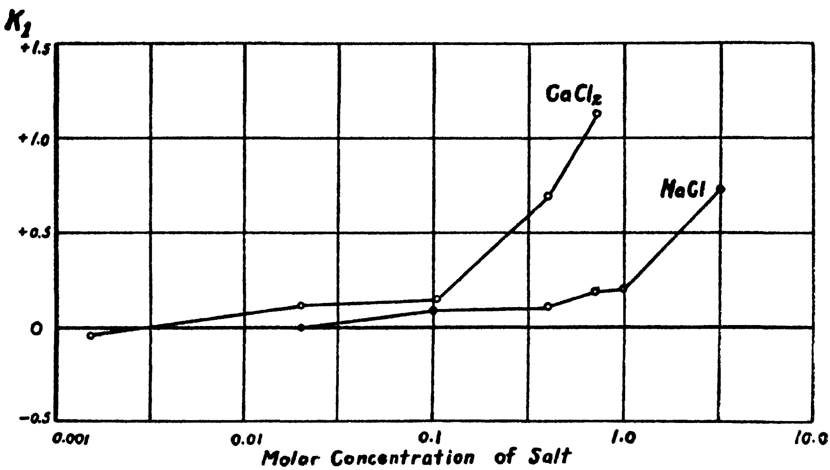

FIG. 4. VIABILITY OF BACT. COLI IN SODIUM AND CALCIUM CHLORIDE SOLUTIONS

base line. We can scarcely assume that the velocity of a lethal reaction is inhibited by certain concentrations and accelerated by other concentrations of one of the reacting substances. We are not aware that chemical dynamics of homogeneous systems can be harmonized with such conditions. The curve, presumably with an origin when both x and y are 0,[14] suggests smooth and continuous gradations for values of K_1 from 0, through negative to progressively increasing positive values. It therefore implies

[14] Because in pure water ($x = 0$) the bacteria maintain themselves in practically undiminished numbers for twenty-four hours.

that there is no sharp discontinuity between such effects of a reagent as result in acceleration of the reproductive rate to such as markedly accelerate the rate of mortality. It might be assumed that the effect of a toxic agent on *Bact. coli* becomes manifest because mortality is the consequence of a stimulation of reproduction to a degree which is incompatible with maintenance of viability (i.e., if the fundamental reactions of stimulation and toxicity are identical); but such a view would be extremely far-fetched except perhaps in connection with the influence of temperature. If it be considered that this assumption is unwarranted, it becomes necessary to consider that the action of a toxic substance upon a bacterial cell is not explicable in terms of a simple type of chemical reaction.[15] The course of a disinfection process is the algebraic resultant of stimulating effects of the toxic agent upon reproduction and of truly toxic effects measured by accelerations of mortality rates. Considered in this light, the time curve of a mortality process may lie at any position between the extremes, i.e., the time curves of the stimulating and of the intoxicating chemical reactions. Variations in the shape of this resultant curve are therefore to be expected with the same as well as with different concentrations of toxic agent.

Certain data which we have cited from our experiments indicate a general concordance between the courses of disinfection by calcium chloride and of bi- or tri-molecular reactions. It is not our aim, however, to contend that the course of a disinfection process necessarily or even generally simulates that of a bi- or tri-molecular chemical reaction. We wish merely to emphasize

[15] By making additional assumptions, especially by assuming reaction in a heterogeneous system in which the number of phases is variable, it may be possible to deduce the equation of such a curve. If the disinfection of a bacterial suspension by $CaCl_2$ is treated as a reaction in a heterogeneous instead of a homogeneous system and if the course of the process is found in accord with the logarithmic law, the significance of the finding becomes radically different from that which has been generally placed upon it. It may mean that in the changes which lead to loss of viability a number of reactions (physical or chemical or both) are involved and that the slowest (and determining) one may be of the nature of a diffusion reaction or of any one of many kinds of multi- or inter-phase reactions (vide Taylor, 1924). The precise treatment of these conditions cannot be conducted with our data.

that although disinfection processes generally give the logarithmic curve and are approximately described by the equation of a unimolecular reaction, under certain conditions—particularly in the presence of certain mild disinfectants—the course of the process may more closely approximate that of a multimolecular reaction.[16]

In the present state of knowledge, the time curve of a disinfection process remains as a valuable tool in the measurement of toxicity but not as a guide to the chemistry or physics of the fundamental reaction between cell or cell constituents and toxic agent. Conclusions to the effect that the time curve is always, necessarily, logarithmic appear not to be in accord with experimental findings. The exceptions from the logarithmic course which have been reported here lead to the conclusion that the mechanism of a disinfection process is probably highly complex. On the other hand the observed variation can be quite plausibly explained on chemical grounds without the introduction of the assumption of biological variability.

VIII. GENERAL CONCLUSIONS

1. The mortality of *Bact. coli* in solutions of NaCl and $CaCl_2$ and in distilled water of varying pH follows a generally logarithmic course and may be roughly described by the equation of a unimolecular chemical reaction, as has been shown to be the case for other processes of disinfection.

2. This relationship is not, however, a close or an exact one. Like most other students of this problem, we find, on close analysis, more or less marked deviations from the logarithmic curve. With NaCl the rate of reaction seems to increase at first. With both salts, and with acid and alkali, it gradually decreases as the experiment proceeds through its later stages.

3. Where disinfection does not follow a logarithmic course and is not to be described by the unimolecular equation, the course of the process may sometimes be described by the equation of a

[16] It is significant to recall that from a recalculation of Chick's data on phenol disinfection, Watson (1909) concluded that the reaction was of at least the seventh order.

bi-, tri-, or higher multimolecular reaction. Such an explanation may render unnecessary the assumption of variability in biological resistance to account for deviation from a logarithmic mortality curve.

4. When used in sufficiently low concentration, NaCl and $CaCl_2$ may be not only without toxic properties for *Bact. coli*, but may even stimulate the organisms to increased growth and reproduction. This observation is in harmony with the conclusions of Richet, Hotchkiss, Madsen and others and appears to be an illustration of a general biological principle. For a series of concentrations of $CaCl_2$ acting upon suspensions of *Bact. coli* a curve may be plotted which shows continuous gradations between intoxicating and stimulating effects.

5. From an analysis of the time curves of disinfection processes it appears that the reaction between disinfectant and cell or cell constituents is probably highly complex; and that while the logarithmic curve best expresses its general rate, the correspondence is not, and cannot be expected to be, always a close one.

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VIABILITY OF BACTERIUM TYPHOSUM IN ICE CREAM

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The experimental results here reported were obtained in connection with a study on the effect of carbon dioxid on typhoid bacteria in ice cream. After the desired information on this point was secured, the ice cream was kept for the purpose of determining how long the typhoid germs would survive in ice cream.

One gallon of ice cream mix was prepared, which contained 12 per cent sugar, 12 per cent solids other than fat, and 10 per cent fat. The mix was sterilized, inoculated with *Bacterium typhosum*, and then incubated until the bacterial count was about 25,000,000 per cubic centimeter. It was then frozen and immediately stored in the hardening room. The temperature in this room fluctuated between 8 degrees below zero and 8 degrees above, but for the most part was about $-4°F$. The ice cream was prepared on January 11, 1922, and was held under the above conditions until all the ice cream was used up by sampling, the last sample being taken on May 20, 1924. Samples for bacteriological examination were taken from the mix just before it was frozen, from the freshly frozen cream, and then at varying intervals during the two years and four months of storage. The bacterial counts are recorded in table 1.

The original culture, as well as the cultures which were recovered from each sample of the ice cream were subjected to agglutination and other laboratory diagnostic tests. These cultures were also tested for us by Mr. Luther Thompson. The culture obtained from the last sample, when the ice cream was two years and four months old, was tested by the authors, by Mr. Thomp-

son and also by Prof. H. M. Weeter.[1] Positive *Bacterium typhosum* tests were obtained in all cases.

Just before freezing the mix gave a count of 25,000,000 bacteria per cubic centimeter. About an hour after freezing, the ice cream gave a count of 51,000,000 per cubic centimeter. This apparent increase was probably due to the breaking up of the bacterial clumps. After five days of storage the count was reduced about 90 per cent and in twenty days the reduction was more than 95 per cent. In 134 days approximately one germ out of every two hundred survived. At the end of one year about one germ out of every thousand survived, and at the end of two years one germ out of every ten thousand survived. After two years and four months of storage living typhoid germs were still

TABLE 1

Persistence of Bacterium typhosum in ice cream

SAMPLES TAKEN	TYPHOID BACTERIA PER CUBIC CENTIMETER OF ICE CREAM
Before freezing	25,000,000
Freshly frozen	51,000,000
5 days old	10,000,000
12 days old	7,000,000
20 days old	2,200,000
70 days old	660,000
104 days old	900,000
134 days old	210,000
165 days old	640,000
170 days old	711,000
200 days old	60,000
260 days old	57,000
290 days old	53,000
342 days old	51,000
430 days old	30,000
544 days old	13,000
648 days old	11,000
2 years	6,300
2 years, 4 months	Living typhoid germs present

[1] We are greatly indebted for the tests to Mr. Luther Thompson, formerly of the University of Illinois, now with Mayo Brothers, Rochester, Minnesota, and to Professor H. M. Weeter, Medical School, Louisville, Kentucky.

present. Accurate count at this time was impossible on account of contamination. It will be observed in table 1 that the samples taken when the ice cream had been in storage for 165 days gave higher counts than the previous samples. To check this point, another set of samples was taken five days later which again gave similar counts. An inquiry brought out the fact that one of the attendants had removed the experimental ice cream a few days before to an adjoining room for an hour and one-half. This room had a temperature of 40°F. The ice cream did not melt. Whether there was any multiplication of the germs at this time could not be determined.

The counts given in the accompanying table indicate that there was a continual decrease in the number of the typhoid germs. The decrease was rapid at first but gradually slowed down. A small percentage of the germs were able to withstand the adverse conditions for a long time. The experiment had to be terminated at the end of two years and four months because all the ice cream was used up by sampling. Some of the typhoid germs were still living at this time and there is every reason to believe that they would have survived even longer.

It is evident that low temperature such as is used for the storage of ice cream can not be relied upon to destroy typhoid germs in ice cream. This had already been demonstrated by others (1).

Why some of the typhoid germs were able to survive so much longer than others is an interesting question still open for further investigation. From other data not given here it was found that the nature of the medium in which the germs are kept is a contributing factor.

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A NOTE ON THE SPIROCHAETES OF TERMITES

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The original observations of spiral organisms in the intestines of termites or "white ants" were made by Leidy (1877), who noted that the gut of *Reticulitermes flavipes* ("*Termes flavipes*") was distended with material consisting largely of infusorial and other parasites. Of these parasitic forms, Leidy identified three as animal and two as vegetable in character. One of these latter he termed a spirillum, "probably *Spirillum Undula.*"

In a later study of the parasites of termites, Leidy (1881) called the spiral organisms "vibrios" and declared they most nearly resembled the *Vibrio serpens* of Müller. Leidy gave these spiral forms the name *Vibrio termites* and described them as rectilinear and regularly undulant with 3 to 6 waves, commonly stationary and undulating more or less rapidly, but often advancing or receding with variable rapidity, sometimes becoming quiescent. Occasionally they were observed to bend at an obtuse angle while continuing to undulate. The smallest individuals were straight but in motion became bent in the segment of a circle; not infrequently they adhered together by one end, thus forming radiating groups.

Other observations on spiral organisms in termites have been reported by Grassi and Sandias (1893–1894), who recorded spirilla in European termites—*Kalotermes flavicollis* and *Reticulitermes lucifugus* ("*Termes lucifugus*").

The latest report on organisms of this type in termites has been made by Dobell (1910–1913), who noted them in the course of certain studies on parasitic protozoa from Ceylon.

To these spiral organisms Dobell gave the name *Spirochaeta termitis*, Leidy, as he concluded that the vibrios described by the earlier worker were undoubtedly spirochaetes or treponemata. Dobell observed in his termites two types of spiral organisms, one of which was much larger than the other and the description of which is identical with the *Treponema minei* of Prowazek. These organisms Dobell reported as varying from 20 to 60 micra long by 0.5 micron in width, pointed at both ends, possessing no flagella or crista, and actively motile. When stained by Giemsa stain these organisms take a uniform pink and occasionally exhibit a granular structure.

With these observations in mind, it occurred to the author that it might be of interest to examine other species of termites, as a considerable number of species were immediately available in this laboratory. In this way it was hoped to get a better idea as to the distribution of spiral organisms among the various families of termites and to study more carefully the morphology of such organisms as might be observed. To do this it appeared necessary to examine fresh preparations, made by emulsifying the material contained in the gut of the termites in a saline solution, by dark field illumination and to devise a method of staining that would permit careful study of smear preparations of the same material.

FIG. 1. A REPRESENTATIVE FIELD OF A SMEAR PREPARATION MADE FROM THE STOMACH CONTENTS OF NASUTITERMES MORIO

A similar abundance of spirochaetes will be seen in smears made from all the species examined.

FIG. 2. A LIMITED FIELD OF A PREPARATION OF TERMOPSIS NEVADENSIS

Note the extreme size of some of the spirochaetes seen in this species

FIG. 3. ANOTHER PREPARATION OF TERMOPSIS NEVADENSIS SHOWING THE SMALLER TYPE OF ORGANISM

FIG. 4. ILLUSTRATION OF THE HEAVILY STAINING TYPE OF ORGANISM FOUND IN CRYPTOTERMES BREVIS

FIG. 5. REPRESENTATIVES OF THE TYPE OF SPIROCHAETAE SEEN ONLY IN KALOTERMES SCHWARZI

Note the occurrence of the circular coils

FIG. 6. ANOTHER FIELD FROM THE SAME PREPARATION AS THAT USED IN THE PRECEDING FIGURE

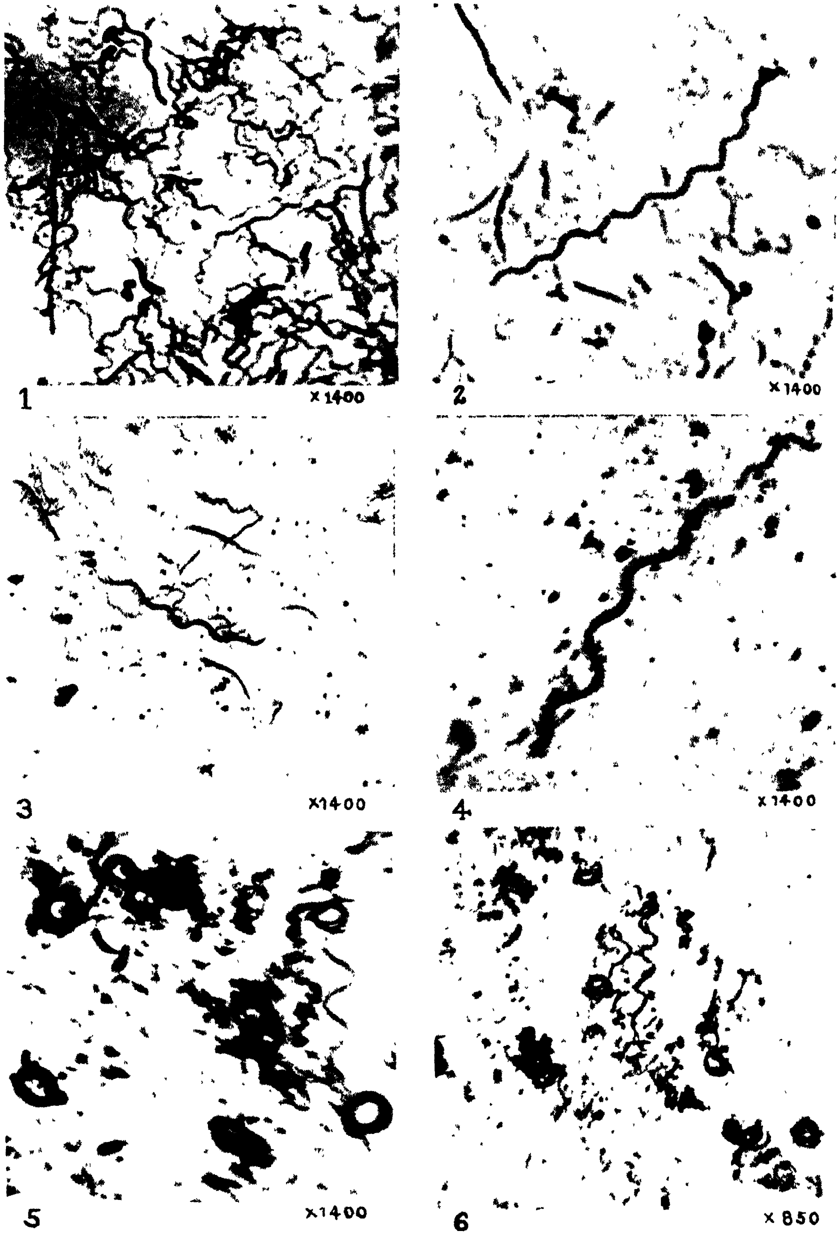

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TABLE 1

Findings in intestinal contents of termites

FAMILY	GENUS	SPECIES	SOURCE	FINDINGS IN INTESTINAL CONTENTS
Kalotermitidae	*Cryptotermes*	*brevis*	Porto Rico	Many heavily staining spirochaetes having 3 to 4 spiral turns, uniformly pointed at both ends and averaging 25 by 1 micra
	Kalotermes	*schwarzi*	Florida	Apparently three types of spirochaetes present One not very numerous, having 4 spiral turns and averaging 40.5 by 1 micra. A larger number of smaller organisms of an average of 25 by 0.5 micra and with 3 spiral turns A small number of another heavily staining organism seen only in tightly wound circular coils
		nigriceps	British Guinea	Examined in stained preparations only Apparently two types of spirochaetes present in considerable numbers varying in size and number of spiral turns
	Termopsis	*angusticollis*	California	Relatively small number of spirochaetes present and these are thin, lightly staining organisms with 2 to 3 spiral curves and averaging 18 by 0.2 micra Actively motile
		nevadensis	Oregon	Two types of spirochaetes only. One is very abundant and has 4 to 12 spiral turns, while the other is much shorter and has only 4 very fine spirals
Rhinotermitidae	*Leucotermes*	*tenuis*	British Guinea	Stained preparation only observed. Many spirochaetes of varying form noted. Some large and taking the stain heavily, others noticeably smaller and staining lightly

Rhinotermitidae......	*Reticulitermes*	*flavipes*	Maryland	An abundance of large heavy staining organisms, 25 by 0.5 micra in size. The ends are pointed and in dark field preparations occasionally one is seen to be motile
		virginicus	Maryland	Apparently the same organisms as seen in *R. flavipes*, except that the majority are actively motile
		hageni	Maryland	An abundance of spirochaetes indistinguishable from those seen in other species native to Maryland
Termitidae............	*Nasutitermes*	*morio*	Porto Rico	A few spirochaetes that stain lightly and average 9 by 0.4 microns. Many organisms with four spiral turns having average dimensions of 19 by 0.5 microns and staining heavily

After considerable experimentation with various stains, the following staining technique was worked out and found to give uniform results:

1. Emulsify the gut contents of the termite in a drop of 0.4 per cent saline and expose to the fumes of osmic acid for thirty seconds.
2. Dry in the air or by very gentle heating.
3. Flood the preparation with a 1:5 solution of 50 per cent carbol fuchsin and 50 per cent aniline gentian violet. Stain sixty seconds.
4. Wash in water, dry and examine.

By this method the background is seen to be a light pink and the organisms much darker as they take the gentian violet dye.

Following the above technique for stained preparations, and by the study of supplemental dark field preparations, ten species of termites, representing three of the four families and six genera were examined, with the results shown in table 1.

From this survey it will be seen that all the species of termites examined are infested with spiral organisms of one or another sort. There does seem to be a difference in the flora, however, as there is considerable variation in the size and staining properties of the spiral organisms observed. It might also seem that there is a difference in the flora when we consider the feature of motility, but we are inclined not to stress this as a differential character, as more extensive observations might indicate motility in all cases. In the cases of those organisms seen to be motile, the motion is that typical of spirochaetes in general, although few of these organisms have been observed to move in any direction other than straight forward or backward. In no stained preparation has there been observed any structure similar to a crista or an undulating membrane and thus far no flagella have been demonstrated.

The accompanying photographs depict typical spiral organisms as observed in various species of termites.

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CLOSTRIDIUM FLABELLIFERUM: A PUTREFACTIVE ANAEROBE WITH BRUSH-LIKE SPORANGIA

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In the summer of 1921 a morphologically peculiar anaerobe was isolated from a table on which hams are handled in a large packing plant in the Midwest. The most striking feature of this organism is the retention by the spore of the vegetative cell, or some part thereof, in the form of a brush-like process made up of innumerable fibrils. This unusual type of sporangium, staining readily by any of the common methods, furnishes a very striking picture especially in older cultures, and contrasts sharply with *C. sporogenes* which rapidly develops free spores. This typical picture has led to such designations as fish tailed and fan tailed spores; hence, the proposed name *Clostridium flabelliferum*. This feature of the organism is constant in favorable media, and in various kinds of media. It is not the result of unusual environment, or unfavorable conditions of growth, but appears to be a normal character of the organism which it has retained for a period of three and one-half years after isolation and culturing in artificial media. A preliminary study has been made of the more important morphological, cultural and biochemical characters and these will now be given as a first step toward a complete description.

MORPHOLOGY

This organism is rod-shaped, with rounded ends, of moderate size, ranging from 3 to 5 by 0.4 to 0.5μ. It forms oval, subter-

minal spores in practically all ordinary media, appearing readily in egg-meat medium within twenty-four to forty-eight hours at 37°C. The sporangium is at first definitely formed, with the edge entire, and well rounded on the distal end (Fig. 1). The spore is larger than the rod, causing one end of the rod to be swollen, with a well-defined tip at the spore end. After two days at 37°C, the sporangium begins to break up into a fan-shaped formation (fig. 1). At first an indefiniteness of outline and a slight flaring of the cell at the end farthest from the spore may be observed. Occasionally the vegetative portion of the sporangium becomes bifurcated, or the longitudinal splitting may give rise to two or more distinct fibrils diverging from the main body of the cell. By the fourth or fifth day, practically all the spores show some degree of this change in sporangium, and the fan-shape is apparent in all degrees of perfection. This condition persists in the egg-meat medium for as long as two months at 37°C. and five months at room temperature, the limits of observation, beyond which time only few free spores, without frayed, fan-like, sporangia are to be found.

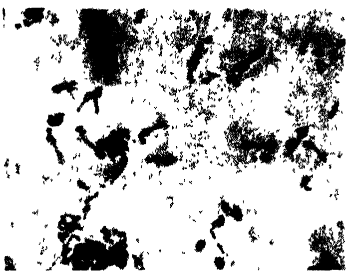

FIG. 1 CLOSTRIDIUM FLABELLIFERUM

Grown in egg-meat medium showing different stages of "fan tail" formation

This peculiar property is in itself distinctive, especially since the character of the spore continues under favorable conditions for a time well past that when the sporangia of other similar anaerobes have become disintegrated and have dropped from the spore, leaving the latter free. It is remarkable, also, that but few so-called free spores have been found in the medium even after two months incubation in meat medium. It seems that the splitting of the sporangium is usually the end of the disintegrative process normally expected in old cultures of spore-bearers. Since this feature is constant, and since it is shown in other culture media, such as plain and glucose broth (four-day cultures), glucose agar (several days old), such fish-tail forms being exactly similar to

those observed in egg-meat medium, this character is deemed of sufficient importance to distinguish the organism from all others. No other anaerobes, or aerobes, have in our experience shown such a peculiarity.

MOTILITY

In ordinary hanging drop preparations, this organism is actively motile in twenty-four-hour egg-meat cultures. The number of flagella has not been determined.

STAINING PROPERTIES

Gram's stain is retained, but is easily decolorized if care is not exercised. When compared with *B. coli* in the same smear, it is decidely Gram-positive when the alcohol is allowed to remain on but a short time. It stains readily with the usual aniline dyes. The frayed sporangium is quite clearly stained when any of the ordinary laboratory stains are used, but shows particularly well with fuchsin. No preliminary treatment with a mordant is required.

COLONIES

Depth colonies. These colonies in one per cent agar shake cultures are wooly and irregular in outline. They resemble *C. sporogenes* colonies. They grow to within about one-half inch of the surface of the agar, the growth in plain and sugar agar being quite luxuriant.

Surface colonies. On the surface of glucose agar (1 per cent glucose, 1.6 per cent agar) growth is obtained within twenty-four to forty-eight hours (in Novy jar by alternate hydrogen and evacuation method) and by the third day present rather coarse, raised colonies with long, intertwining outgrowths. These colonies, too, are similar to those of *C. sporogenes.* In testicular extract agar, the colonies appear tetanus-like, with faint outgrowths, leading to irregular sunbursts in an irregular zone frequently as wide as the diameter of the colony.

CULTURAL CHARACTERS

Egg-meat. In this medium, from which free oxygen was expelled by boiling, growth takes place readily, even when incubated aerobically, within twenty-four hours, with the evolution of slight amounts of gas. Even in this short period of incubation, some few spores are produced, the sporangia of which are fully formed and with edges entire. There is also a very slight odor of putrefaction, which becomes more pronounced on further incubation. The meat shows the usual darkening and breaking up into finer particles such as is shown also by *C. sporogenes* and other putrifiers. This disintegration progresses until half of the medium has been liquefied, when the action is practically stopped. By this time, the meat has become dark brown, with a dirty, amber-colored supernatant liquid.

Milk. Milk, incubated anaerobically under solid paraffin, is readily digested in forty-eight hours. There is at first a soft clot, followed by digestion of the precipitated casein. By the fourth day about half of the medium has been digested, the resulting liquid above the undigested portion being of a dark, amber color. With longer incubation, little or no change takes place.

Gelatin. Plain and glucose gelatin, inoculated and incubated aerobically immediately after autoclaving, are liquefied within twenty-four hours at 37°C. There is no apparent difference between the times when both are liquefied.

Broth. There is rapid and abundant growth in both plain and glucose broth, incubated under anaerobic condition sunder solid paraffin at 37°C. In the latter there is also copious gas production. The growth first appears uniformly distributed throughout the medium but partially settles out after thirty-six to forty-eight hours. The growth becomes abundant and has a tendency to flake out leaving the broth quite clear.

BIOCHEMICAL REACTIONS

Fermentation tests. Preliminary observations on the fermentation of sugars are not entirely conclusive. In the main the

new organism agrees with *C. sporogenes.* Glucose, levulose, galactose, maltose and sucrose are fermented with gas production. Further observations will be necessary before it can be stated whether the two organisms differ in their carbohydrate utilizations.

Glucose-consuming power. In glucose broth containing 0.87 per cent glucose, the sugar was reduced to 0.51 per cent in four days when incubated under anaerobic conditions, at 37°C. *C. sporogenes,* under similar conditions reduced glucose from the same figure to 0.49 per cent in the same time. These figures are so close as not to be significant in separating the two organisms. The quantitative method of Benedict[1] was used.

Peptone-consuming power. The Sorensen test has been shown to be sufficiently accurate to determine relative degrees of the peptolytic power of anaerobes, and was used here to show the peptolytic power of this organism as compared with that of *C. sporogenes.* It has been found, incidentally, that the figures obtained for *C. sporogenes* in this test are surprisingly close to figures reported two years ago by the junior author, under different laboratory conditions and with different reagents, showing that the method is sufficiently accurate for this purpose. Plain peptone-beef extract broth was used. The original medium had a Sorensen figure of 44 (number of cubic centimeters of N/20 NaOH per 100 cc. of medium). After four days growth of the new organism at 37°C, this figure was raised to 116. The difference is 72 according to this scale. *C. sporogenes* gave a figure of 122 when grown under the same conditions: the increase over control is 78. Of the two strains studied therefore, the *C. flabelliferum* had slightly less peptolytic action.

Pathogenicity. This organism is non-pathogenic for the ordinary laboratory animals, namely, mice, guinea pigs and rabbits. A 1:10 suspension of a two weeks old culture in egg-meat was used for the injections, which were made intraperitoneally. No ill effects whatever were noted.

Salt relation. It is of practical interest to note the salt tolerance, with special emphasis on the minimum concentration that

[1] Journal of Bacteriology, 1924, **9**, 13.

will inhibit growth. Tubes of egg-meat medium containing the following concentrations of NaCl were inoculated with the organism and incubated aerobically at 37°C; 1, 3, 4, 6, 8, 12, and 16 per cent. Two series were inoculated, one with *C. sporogenes* and the other with the new organism. After three days incubation, the following conditions were observed:

1. Marked putrefaction, in concentration up to and including 6 per cent NaCl in both series. After twenty days incubation, a slight odor was observed in 8 per cent NaCl. Smears show many vegetative cells in tubes containing as high as 12 per cent salt in spite of the negative odor. Smears from the 16 per cent salt tubes showed occasional vegetative cells. Very few spores were found in any of the salt media, even after two months incubation. This is in agreement with our previous observations that, in general, spore formation either by halophils or other organisms is inhibited by NaCl.

Ham souring experiment. Attempts to produce a "sour ham" by means of *C. flabelliferum* have been negative. A suspension[2] made from an egg-meat culture was injected into one ham and three shoulders about one half hour after the slaughter. The paired ham and shoulder was in each case labelled "control" and was kept, uninoculated, for comparison. All hams and shoulders were hung in a chill room one hour after the inoculation was made and subsequently cured according to the regular packing house routine. Observations were made with a trier after the hams had been in cure for a month and the packing house inspector was unable to detect any sour odor either in the controls or in the inoculated pieces.

This experiment is not considered altogether conclusive. It is known to be rather difficult to produce a typically "sour" ham experimentally. The heaviest inoculation used represented only 0.75 cc. of the original culture and the others considerably

[2] This suspension was made by emulsifying 5 cc. of an old egg-meat culture in 45 cc. of physiological saline, filtering through cotton and centrifuging. The putrid fluid was then poured off and the sediment containing most of the bacterial cells was resuspended in 100 cc. of saline. For injection into the shoulders 3, 7, and 15 cc., quantities were used and 7 cc. for inoculating the ham. Injections were made at the points where experience shows that souring is most apt to occur.

less. The majority of the organisms were in the spore stage and their subsequent development may have been slower than would have been the case with vegetative cells. We are not justified in saying that under certain conditions this organism might not be capable of causing a "sour ham."

SUMMARY

A morphologically distinct species of putrefactive anaerobe has been isolated and studied. It is characterized by the formation of brush-like sporangia and the persistence of these forms even in two-month-old cultures.

It further differs from *C. sporogenes* in the appearance of broth cultures in which the growth rapidly settles out leaving the media clear.

No marked biochemical differences have been noted between the new organism and *C. sporogenes.*

The characters noted above have remained perfectly constant over a period of three years and seem to warrant a new species for which the name *Clostridium flabelliferum* is proposed.

THE MULTIPLICATION OF YEASTS AND YEAST-LIKE FUNGI IN SYNTHETIC NUTRIENT SOLUTIONS

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Numerous papers have been published during the past few years on the propagation of yeasts in inorganic salt-sugar media. Studies in this field are of great import since they may form the bases for a better understanding of the fundamentals of nutrition not only of this interesting group of microorganisms but of the higher organisms as well. The literature on the subject has become rather voluminous. Since it would be impossible to review it in this paper, a review has been prepared for publication in another place. It might be stated, however, that interest in the subject was quickened by the announcement of Wildiers in 1901 that a special substance of unknown chemical composition was necessary for active multiplication of yeasts.

Before presenting the results of our own experimental work, we wish to discuss a few points which seem pertinent after an analysis of some of the reports in this field of research. Such a survey shows that different conceptions of the term growth have been used. These are probably best defined by the methods which have been employed for following the behavior of yeasts in inorganic salt-sugar media. It is quite evident that the methods which have been used have greatly determined the results which have been obtained. A microbiologist sees an evident error in the use of one or two species of yeasts. Several investigators in this field have used but one species in a single medium and generalized to include all of the different species. Some have refuted the data and conclusions from other laboratories secured with a particular species and medium, with data

secured by themselves with another medium and species. Eddy and Stevenson (1920) reported that they investigated some thirty strains of yeast in connection with the development of their yeast test; they did not, however, state whether they used different species or different strains of one or a few species.

It seems necessary, also, to make a sharp distinction between the terms growth, multiplication, propagation, development, proliferation, etc. One must distinguish between data collected by the following methods:

1. Microscopic cell counts
2. Plating in solid media
3. Counting stained smears
4. Weighing increase in yeast mass
5. Measuring the volume of yeast mass
6. Measuring some product of growth or fermentation
7. Nephelometer methods
8. Nitrogen determinations
9. Viability tests on laboratory media

Ide had somewhat the same idea in mind when in reply to a paper by McDonald and McCollum (1921) he pointed out that there were two kinds of proliferation of yeast, one very slow without "bios" and one fast with "bios." Ide claimed that between the two there was such a difference that no one could mistake it once he had seen it.

Some recent work by Henrici (1922) on the bacteria has a significant relation to this discussion. He showed by biometric methods that cells of bacteria grew in size during the so-called lag phase in which it is ordinarily considered that no growth is taking place. In many of the reports in this field of research growth has been followed by means of counting methods. Such methods really depend on cell division or multiplication, functions which may be quite different from growth. It is possible to have growth without cell division. Bachmann (1919) measured carbon dioxide formation as an index of growth. Such a method is accurate if growth and fermentation are always proportional. The work of Henrici, just mentioned, is also sup-

ported by data reported by Clark and Ruehl (1919). They reported striking changes in morphology of bacteria especially during the early stages of growth (from two to six hours). The cells from young cultures of bacteria were generally smaller than those from old cultures. These data indicate that the cell size may increase after multiplication has ceased. While such data on bacteria lend support to this argument, it is fortunate that we have data for yeasts themselves. Slator (1923), after enumerating the various methods that may be used for following the development of yeasts, made the following statement:

> The results obtained by these various methods are sometimes different. For instance, if the growth of yeast in wort is followed, the number by counting stops when the alcohol concentration reaches about 3 per cent, but by centrifuging, growth is shown to continue longer, owing to an increase in the size of the cells. An increase of 20 per cent in amount is often observed after the number has reached a maximum.

A statement from such an authority as Slator indicates the futility of comparing data on "growth" of yeasts which have been secured by different methods. This suggests that some of the confusion and misunderstanding in this field may be due to the lack of a proper understanding of the limitations of the various procedures which have been used.

Then, one may ask whether "growth" is the only criterion of the response of the yeast plant to such a substance as "bios." The term "yeast growth stimulant" might seem to rule out a discussion of this question, but it is apparent that yeast may grow and multiply even though it is not healthy. This fact was mentioned by Wildiers. Wildiers found that yeast would grow slowly in the synthetic media but that the cells were not like normal cells. They were not "healthy." Investigators, especially those in America, have overlooked this factor. German fermentologists have always considered it. A medium which does not permit the development of healthy cells even though it may permit a slow multiplication, is probably not a good one.

We ought also to direct attention to the fact that certain of the vitamin extracts which have been used probably contained food materials of more definite chemical constitution than "bios" is supposed to be, which stimulated growth. It is very difficult to know just how far an extract should be diluted or how it should be prepared perhaps, to contain only "bios" and no other organic matter which would influence growth. The cells of microörganisms are very small. Assume the size of an ordinary yeast cell to be 5μ in diameter. It may be greater or less than this but the above size is not far from an average for *Saccharomyces cerevisiae* which is usually spherical or oval. The amount of solid matter in a cell would be computed as follows:

5μ = 0.005 mm.
Vol. Sphere = $4.1888r^3$.
r^3 = 0.000,000,015,625 cu. mm.
r^3 4.1888 = 0.000,000,065,45 cu. mm. in sphere.
Specific gravity may be assumed to be 1.
0.000,000,065,45 cu. mm. = 0.000,000,065,45 mg.
85 per cent of cell is water. 15 per cent = solid matter.
15 per cent of 0.000,000,065,45 = 0.000,000,009,817,5 mgm. solid matter in a yeast cell 5μ in diameter.

It is easily seen that the vitamin extracts have never been diluted sufficiently to avoid the possible addition of amounts of chemically definite organic matter sufficient to promote growth and multiplication or at least to allow it to start. Once it has started other factors may enter.

EFFECT OF BACTERIAL CONTAMINATION

Not many data are available on the effect of contaminating bacteria on the growth of yeast in the pure synthetic solutions. It is interesting, however, to note that Williams reported better growth of this yeast when the solutions became contaminated with bacteria. However, from the description of his yeast method, which Williams published in 1920 and which is apparently his last contribution to the subject, it is evident that he did not have a pure culture. He weighed 0.300 gram of fresh Fleischmann's yeast (small cake in tin foil) taken from the center of the cake. It is well known to bacteriologists that Fleischmann's

yeast as used by Williams, is contaminated with bacteria. Consequently Williams' data may have been greatly influenced by contaminating bacteria. This influence may either be antibiotic or symbiotic depending upon the type of bacteria which happened to be present. Many of the investigators have not stated specifically just how they protected their media from bacterial contamination or just how they determined that bacteria were not present in their culture flasks. Some of these factors are now being studied in this laboratory.

EXPERIMENTAL

A study of the literature indicates that only a few yeasts have been used in the work on accessory substances in yeast metabolism. Most of the work has been done with *Saccharomyces cerevisiae* from Fleischmann's compressed yeast. In but a few cases have other species been used. Fulmer and Grimes (1923) used *Saccharomyces cerevisiae*, *Torula sphaerica*, and a *Mycoderma*. MacDonald (1922) used five different strains from probably not over two species. A microbiologist sees an evident weakness in generalizing from data secured with but one or a few species of yeasts. However, it is realized that if a standard biological procedure for testing for the presence of accessory substances in materials should be devised, probably a single species of yeast would have to be accepted as the standard test organism.

In this investigation we were interested in the following: First, the use of media containing no "bios" or other accessory substance; and secondly, whether we could secure the growth of a great many different species of yeasts in such media with the technic proposed by other investigators. It must be pointed out that all of our inoculations were large. The question of single cell inoculations is now being studied.

While several different media could have been selected Fulmer and Nelson's Medium F incubated at 37°C. Naegeli's and Fermi's solutions were chosen. Fulmer's Medium F was selected because it seemed to have resulted from a serious attempt, based on considerable experimental work, to find a suitable

medium for yeasts. It did not result from a mixture of chemical substances which were assumed to be suitable. Medium F had the following composition[1] and was handled exactly as by Fulmer and Nelson: Ammonium chloride, 0.188 gram; calcium chloride, 0.100 gram; sucrose, 10.00 grams; dibasic potassium phosphate, 0.10 gram; dextrin, 0.60 gram; distilled water, 100.00 cc. and temperature 37°C. All chemicals were the purest obtainable. The ammonium chloride content of this medium was adjusted for incubation at 37°C. Some of our confirmatory cultures were held at 30°C. This medium when prepared according to the above formula, had a flocculent precipitate of insoluble matter probably calcium phosphate. This was left in the culture flasks since its disappearance served as one criterion of multiplication. When the yeasts multiplied and grew in this medium acids, which dissolved the precipitate, were often formed from the sugars. The medium was placed in clean, sterile Erlenmeyer flasks in amounts of 49 cc. and sterilized in the autoclave. To start the cultures suspensions of the yeast from glucose agar slants were prepared in physiological sodium chloride solution and a small amount added to the first flask. After that the technique of Fulmer and Nelson was strictly adhered to. One cubic centimeter of the culture in the flasks was used for inoculating a sterile flask. Incubation periods of three days at 37.5°C. were used. Frequently plates were made from these flasks in order to insure that the cultures were pure and free from contaminating bacteria and that there was a distinct increase in the numbers of cells. This method, then, was really a measure of multiplication ability and not of growth in the strict sense. Some of the pure cultures grew very slowly in both the test medium and on the glucose agar plates. Culture no. 41, *Torula rubra*, for instance, was a good example. This suggests the necessity of making adequate observations before stating that no growth occurred.

The following pure cultures were used in the preliminary qualitative work. Some of the cultures were lost and were not included in the quantitative studies.

[1] This is the formula for incubation at 30°C.

1. Burgundy wine
2. Champagne yeast
3. *Cryptococcus agregatus*
4. *Cryptococcus glabratus*
5. *Cryptococcus Ludwigii*
6. *Debaromyces tyrocola*
7. *Endomyces albicans*
8. *Endomyces javanensis*
9. *Monilia tropicalis*
10. *Mycoderma rugosa*
11. *Mycoderma vini*
12. *Oidium albicans*
13. Yeast from oysters—Hunter
14. *Schizosaccharomyces Pombe*
15. *Parasaccharomyces Ashfordii*
16. *Parasaccharomyces Thomasii*
17. *Pichia farinosus*
18. *Pichia membranaefaciens*
19. Red yeast (Snow)
20. *Saccharomyces albus*
21. *Saccharomyces anomolus*
22. *Saccharomyces capsulans*
23. *Saccharomyces carlsbergensis*
24. *Saccharomyces cerevisiae*
25. *Saccharomyces ellipsoideus*
26. *Saccharomyces hominis*
27. *Saccharomyces intermedius*
28. *Saccharomyces logos*
29. *Saccharomyces mandshuricus*
30. *Saccharomyces marxianus*
31. *Saccharomyces neoformans*
32. *Saccharomyces Pastorianus*
33. *Saccharomyces* spec—Plimmer
34. *Torula colliculosae*
35. *Torula communis*
36. *Torula cremoris*—Hammer
37. *Torula datilla*
38. *Torula glutinis*
39. *Torula humicola*
40. *Torula mucilaginosa*
41. *Torula rubra*
42. *Torula sphaerica*
43. *Willia anomala*
44. *Willia belgica*
45. *Willia saturnus*
46. *Zygosaccharomyces bisporus*
47. *Zygosaccharomyces chevalieri*
48. *Zygosaccharomyces mandshuricus*
49. *Zygosaccharomyces pastori*
50. *Zygosaccharomyce* Priorianus

51–72. 22 cultures of yeast-like fungi from sore throats (Tanner and Dack, 1924); probably Monilia

Our first object was to determine whether the yeasts would multiply actively in the synthetic media adopted. This was done by inoculating the medium in 50 cc. quantities from a salt suspension of cells grown on agar slants. Growth was determined by the preparation and counting of glucose agar plates, by appearance and by the odor. Some of the species had a tendency to climb the walls of the container after growth had progressed. This made a good additional criterion of growth and multiplication. Several species formed pigments also.

FULMER AND NELSON'S MEDIUM F

The first inoculation into Fulmer and Nelson's medium was made October 1, 1923. Transfers were made every three or four days until March 28, 1924. Twice during this period one week elapsed between subculturing (Thanksigiving and Christ-

mas). When it was decided to include Nägeli's and Fermi's solutions in the work a period of one week between sub-cultures was decided upon. There was a total of about 57 transfers. Early in the fall of 1924, the yeasts were again put in Fulmer and Nelson's medium. These cultures were again started from agar slants. The counts, however, shown in the tables were made on a series of flasks which were being carried along to determine whether these media would support the yeasts. All of them grew although there were differences in the intensities and rates of growth. At frequent intervals the yeasts were counted using glucose agar to determine the multiplication rate. When flasks which seemed to be poor in growth, were left at room temperature, growth was frequently accelerated, showing that 37°C. was not an optimum temperature for all of the cultures used. Although no special experiments were carried out to prove it, it could be seen that after several transfers in Fulmer and Nelson's medium, the cultures grew more quickly as if they were accustomed to it. Finally to secure quantitative data, one set of flasks was chosen for enumeration of the number of cells. These data are shown in table 1.

Examination of the data in this table leads to the conclusion that yeasts are able to multiply in the pure inorganic salt-sugar media of Fulmer and Nelson if the initial inoculation is sufficiently heavy. The multiplication in this medium was markedly superior to that in Nägeli's or Fermi's medium. Eddy and Stevenson also found Fulmer's medium to be better than Nägeli's. The quantitative data shown in the several tables should probably not be taken to show much more than that these budding fungi were able to multiply in the mediums used. The lower counts recorded for some species were probably due to slow growth.

All flasks displayed good multiplication and were grown for a period of about four weeks in the fall of 1924, transfers being made every week except with the last set which was allowed to run for eleven days. As a rule the yeast growth settled to the bottom of the flasks producing a layer of heavy white sediment. On agitation a milky suspension was formed. In some cases the

sediment was more granular or flocculent than in others. Some of the yeasts produced their natural pigment; in two cases the surface of the medium was partially filled with gas bubbles. All of this might be taken as indicating good multiplication. Yeasty odors were produced in all except four cases, namely, 39, 42, 44 and 46.

Another experiment was made to determine the effect of adding accessory substances to medium F. Two sets of flasks prepared as outlined above were sterilized. To one set 0.5 cc. of sterile yeast water prepared according to the instructions of Fred, Peterson and Davenport (1920) was added. Counts were made over a weeks time and the results plotted to determine the slope of the curves. The results of this experiment were not convincing. In some cases there seemed to be a real stimulation on the addition of the yeast water, as if some accelerating substance had been added, while in other cases the slope of the curve was less pronounced and might have been due to the food substances which were present in the yeast water and not so much to an accelerating substance. These curves will not be published at the present time since more work will be done on this question.

Those who have been unable to confirm Fulmer's work have suggested that he used impure ingredients in his medium (Robertson and Davis, 1923). These authors suggested that the cane sugar might be a source of bios. To meet this criticism Fulmer and Nelson extracted some cane sugar for seven days with 95 per cent alcohol in a continuous extractor. Media prepared from this sugar did not give poorer growth nor did the alcoholic extract increase growth when it was added to media. Fulmer and Nelson used 95 per cent alcohol for the extraction. It might be stated that Williaman and Olsen found that 95 per cent alcohol was a poor solvent for bios and that an 80 per cent solution was best. Fulmer, Nelson and White (1923) in a fine piece of work brought very strong experimental data to support their former conclusions that "bios" is not necessary or if necessary may be synthesized by the cell. They prepared methose, a carbon-nitrogen compound of wholly synthetic origin, and found that it could be substituted in place of cane sugar in their

TABLE 1

Showing progress of multiplication in Fulmer and Nelson's Medium F

CULTURE NUMBER	NAME OF YEAST	INITIAL COUNT PER CUBIC CENTIMETER	TWO-DAY COUNT PER CUBIC CENTIMETER	FIVE-DAY COUNT PER CUBIC CENTIMETER	APPEARANCE AT ELEVEN DAYS
1	Burgundy wine	10,000	1,500,000	4,000,000	Heavy growth on bottom
2	Champagne yeast	25,000	120,000	3,500,000	Humpy growth on bottom
3	*Cryptococcus agregatus*	11,000	3,000,000	5,100,000	Heavy growth on bottom
4	*Cryptococcus glabratus*	200,000	2,100,000	2,600,000	Good growth, light, flocculent
5	*Cryptococcus Ludwigii*	600	600,000	2,500,000	Heavy growth on bottom
7	*Endomyces albicans*	300,000	3,000,000	7,700,000	Heavy growth on bottom
8	*Endomyces javanensis*	30	(Slow growth)	5,000,000	Good growth, light, flocculent
9	*Monilia tropicalis*	300,000	15,000,000	2,500,000	Heavy growth, adheres to bottom, milky when agitated
10	*Mycoderma rugosa*	7,600	2,000,000	500,000	Heavy growth on bottom
11	*Mycoderma vini*	200,000	13,500,000	2,500,000	Heavy growth, adheres to bottom, milky when agitated
13	Yeast from oysters—Hunter	3,000	2,300,000	10,000,000	Humpy growth, light, flocculent
15	*Schizosaccharomyces Ashfordii*	800	430,000	3,600,000	Excellent growth, suspended coarse flocs
17	*Pichia farinosus*	200,000	3,200,000	50,000,000	Heavy growth on bottom
18	*Pichia membranaefaciens*	600	700,000	7,500,000	Heavy growth on bottom
19	Red yeast (Snow)	420	2,250,000	5,200,000	Heavy growth, adheres, milky on agitation, reddish color
20	*Saccharomyces albus*	200,000	950,000	400,000	Heavy growth on bottom
21	*Saccharomyces anomolus*	250,000	20,000,000	20,000,000	Heavy growth, bubbles of gas
22	*Saccharomyces capsulans*	800	300,000	100,000	Good growth, light, flocculent
23	*Saccharomyces carlsbergensis*	1,020	(Slow growth)	100,000*	Heavy growth on bottom
24	*Saccharomyces cerevisiae*	960	200,000	500,000	Heavy growth, slight yellow color
25	*Saccharomyces ellipsoideus*	3,500	4,400,000	1,700,000	Lumpy growth on bottom
26	*Saccharomyces hominis*	14,000	500,000	5,000,000	Lumpy growth on bottom
27	*Saccharomyces intermedius*	5,200	4,700,000	1,100,000	Heavy growth on bottom

28	*Saccharomyces logos*	7,000	100,000	1,050*	Coarse flocs covered bottom
29	*Saccharomyces mandshuricus*	12,000	75,000,000	3,500,000	Heavy growth on bottom
30	*Saccharomyces marxianus*	660	1,900,000	9,500,000	Good growth, light, flocculent
31	*Saccharomyces neoformans*	360	1,700,000	100,000	Growth on bottom, one mold, also, was not present when last plate was made
32	*Saccharomyces Pastorianus*	900	6,000,000	1,100,000	Heavy growth on bottom
33	*Saccharomyces* spec—Plimmer	25,000	3,300,000	2,000,000	Good growth, light, flocculent
34	*Torula colliculosae*	22,000	36,000,000	7,300,000	Heavy growth on bottom
35	*Torula communis*	150	(Slow growth)	200,000*	Heavy growth on bottom
36	*Torula cremoris*—Hammar	300,000	72,000,000	1,700,000	Excellent growth, coarse flocs, suspended
37	*Torula datilla*	7,500	300,000	200*	Good growth, light, flocculent
38	*Torula glutinis*	1,800	(Slow growth)	100,000*	Good growth, light, flocculent
39	*Torula humicola*	300,000	15,000,000	1,200,000	Slight growth, surface mold-not present at five-day count
40	*Torula mucilaginosa*	1,850	5,600,000	3,200,000	Heavy growth, reddish color
41	*Torula rubra*	(Too many)	350,000	150,000*	Heavy growth, reddish color
42	*Torula sphaerica*	5,200	165,000,000	100,000	Slight growth, surface mold-not present at five-day count
43	*Willia anomala*	50,000	34,000,000	5,300,000	Heavy growth, bubbles of gas
44	*Willia belgica*	10,000	1,400,000	900,000	Slight growth bottom, mold, not present at five-day count
45	*Willia saturnus*	225,000	20,000,000	19,000,000	Heavy growth, slight red tinge
46	*Zygosaccharomyces bisporus*	7,800	4,500,000	3,100,000	Slight growth bottom, mold, not present at five-day count
47	*Zygosaccharomyces chevalieri*	8,000	8,000,000	900,000	Heavy growth on bottom
48	*Zygosaccharomyces mandshuricus*	200,000	21,000,000	3,000,000	Heavy growth on bottom
49	*Zygosaccharomyces Pastori*	200,000	1,600,000	1.600,000	Heavy growth on bottom
50	*Zygosaccharomyces priorianus*	9,300	5,000,000	900,000	Heavy growth on bottom
51–72	Twenty-two cultures of yeast-like fungi from pathogenic throats gave luxuriant growth in this medium				

* Eleven-day count.

medium. The yeast was able to grow in this solution. Funk and Freedman (1923) also reported a growth-promoting factor for yeasts in cane sugar. While a yeast growth promoting substance may be present in some sugars, one may probably select one which, after study, will be satisfactory for work on yeast multiplication. The sucrose used in these investigations when used in synthetic media would not support the growth of yeast in small inoculations. We know of no other test that may be applied to a substance.

One of the favorable factors for Fulmer and Nelson's medium is probably the insoluble material. This serves as a buffer and removes acids which are formed from the sugar. In many of the synthetic and non-synthetic nutrient solutions developed in former days for the propagation of yeast, a buffer or "neutralizer" was added. It was found that a considerable increase in yield followed the presence of such a compound. As stated above, Fulmer and Nelson's Medium F is the result of considerable study of the effect of the several ingredients. One possible disadvantage, but not a serious one, is the dextrin which causes some inconvenience in preparation.[2] All of the cultures of yeast-like fungi from sore throats gave abundant growth in Medium F.

GROWTH IN FERMI'S MEDIUM

As the investigation progressed it was decided to include several other media. Fermi's medium was introduced first of all. This was prepared as follows: Distilled water, 1000 cc.; magnesium sulfate, 0.2 gram; dibasic potassium phosphate, 1 gram; ammonium hydrogen phosphate, 10 grams; glycerol, 45 grams. It was autoclaved at 15 pounds for ten minutes. The technic of inoculation and observation was identical with that

[2] Fulmer and his colleagues have developed four synthetic media, C, D, E and F. Mediums E and F are especially useful in the cultivation of the budding fungi. Medium E differs from Medium F in the absence of dextrin. Fulmer has advised the authors that Medium E is about as satisfactory as Medium F from the standpoint of yeast crop. Medium E has the advantage that one does not have to bother with the dextrin. Professor Fulmer sent us the following formula for adjusting the ammonium chloride content to the temperature of incubation: 0.188 plus (x°C. − 30°C.) 0.0032.

described for Fulmer and Nelson's medium. In general, multiplication was less abundant in Fermi's medium than in Fulmer and Nelson's. Examination of the data in table 2 indicates an evident difference in ability to utilize the available foods in Fermi's medium. It will be seen that *Saccharomyces carlsbergensis*, *Saccharomyces logos*, *Saccharomyces mandshuricus*, *Torula communis*, *Zygosaccharomyces mandshuricus*, and *Zygosacharromyces Pastori* did not find Fermi's medium as suitable for multiplication as did certain of the other yeasts. This fact is evidence that one should not select a species of yeast and a medium without first determining the behavior of the yeast in the medium selected. It is also necessary to know the temperature relations of the species in order that cultures may be held at the optimum temperature for growth and multiplication.

In order to determine whether the cells were still viable in the cultures which gave no multiplication in Fermi's medium, 0.5 cc. of yeast water, prepared according to the instructions of Fred, Peterson and Davenport, was added to each of these flasks. It was assumed that the cells might be viable but not able to multiply on account of the lack of the supposed hypothetical bios, or for some other reason. When no multiplication resulted after the addition of yeast water, it could be assumed that there were no viable yeast cells present. In these cases more viable cells were put into the nutrient solution plus yeast water. Several attempts to induce these species to multiply rapidly in the plain synthetic nutrient solution were without results. After the addition of yeast water *Saccharomyces carlsbergensis* and *Saccharomyces cerevisae* showed increased multiplication. When yeast water and more viable cells were added, *Cryptococcus glabratus* and *Saccharomyces intermedius* showed abundant multiplication while the other yeasts showing slow multiplication in table 2 did not undergo a stimulation. The cultures from sore throats gave less vigorous growth in Fermi's medium than in Medium F.

TABLE 2

Showing progress of multiplication in Fermi's medium

CULTURE NUMBER	NAME OF YEAST	INITIAL COUNT PER CUBIC CENTIMETER	TWO-DAY COUNT PER CUBIC CENTIMETER	FIVE-DAY COUNT PER CUBIC CENTIMETER	APPEARANCE AT SEVEN DAYS
1	Burgundy wine	3,000	1,700	3,500	Liquid clear, stringy growth in bottom
2	Champagne yeast	3,000	15,000	3,000	Clear
3	*Cryptococcus agregatus*	4,000	47,000	81,000	Cloudy, light sediment
4	*Cryptococcus glabratus*	300,000	50,000	7,500	Clear
5	*Cryptococcus Ludwigii*	12,000	460,000	194,000	Cloudiness, decided sediment
7	*Endomyces albicans*	5,000	500,000	400,000	Cloudiness, decided sediment
8	*Endomyces javanensis*	1,500	180,000	3,600	Clear liquid, stringy growth in bottom
9	*Monilia tropicalis*	12,000	460,000	400,000	Cloudy with viscid sediment
10	*Mycoderma rugosa*	2,000	110,000	150,000	Cloudy, light sediment
11	*Mycoderma vini*	10,000	150,000	191,000	Cloudy, light sediment
13	Yeast from oysters—Hunter	12,000	510,000	1,120,000	Cloudy, light sediment
15	*Parasaccharomyces Ashfordii*	4,000	80,000	260,000	Cloudy, granular sediment
17	*Pichia farinosus*	1,000,000	3,500,000	7,000,000	Milky liquid, flocculent sediment
18	*Pichia membranaefaciens*	54,000	200,000		
19	Red yeast (Snow)	3,000	250,000	154,000	Cloudy, light sediment
20	*Saccharomyces albus*	2,000	138,000	120,000	Cloudy, light sediment
21	*Saccharomyces anomolus*	32,000	2,400,000	2,350,000	Milky liquid, flocculent sediment
22	*Saccharomyces capsulans*	3,000	500,000	12,000,000	Strong clouding, sediment
23	*Saccharomyces carlsbergensis*	5,000	(Slow growth)	(Slow growth)	Clear
24	*Saccharomyces cerevisiae*	5,000	128	50	Clear
25	*Saccharomyces ellipsoideus*	200,000	48,000,000	17,000,000	Strong clouding, sediment

26	*Saccharomyces hominis*	12,000	1.500.000	2,820,000	Cloudy, light sediment
27	*Saccharomyces intermedius*	6,000	300	75	Clear
28	*Saccharomyces logos*	1,000	(Slow growth)	(Slow growth)	Clear
29	*Saccharomyces mandshuricus*	200,000	(Slow growth)	(Slow growth)	Clear
30	*Saccharomyces marxianus*	250,000	900	200	Clear
31	*Saccharomyces neoformans*	3,000	36,000	100,000	Cloudy, light sediment
32	*Saccharomyces Pastorianus*	7,000	108,000	220,000	Cloudy, light sediment
33	*Saccharomyces spec*—Plummer	2,000	30,000	7,000	Cloudy, light sediment
34	*Torula colliculosae*	150,000	2,000	850	Clear
35	*Torula communis*	900	(Slow growth)	(Slow growth)	Clear
36	*Torula cremoris*—Hammar	250,000	50,000	3,000	Clear
37	*Torula datilla*	10,000	5,000	1,100	Slight sediment, apparently no growth
38	*Torula glutinis*	500,000	50,000	36,000	Slight cloudiness, stringy growth in bottom
39	*Torula humicola*	30,000	2,700,000	530,000	Decided cloudiness, sediment
40	*Torula mucilaginosa*	20,000	262,000	300,000	Cloudy, light sediment
41	*Torula rubra*	1,500	500,000	50,000,000	Strong clouding, sediment
42	*Torula sphaerica*	260	1,100	1,230	Apparently no growth
43	*Willia anomala*	1,000,000	480,000	6.000,000	Milky liquid, large growth
44	*Willia belgica*	78	25,000	725	Apparently no growth
45	*Willia saturnus*	30,000	214,000	468,000	Milky liquid, large growth
46	*Zygosaccharomyces bisporus*	250,000	7,000	600	Clear
47	*Zygosaccharomyces chevalieri*	225,000	8,000,000	24.000.000	Strong clouding, sediment
48	*Zygosaccharomyces mandshuricus*	5,000	850		Clear
49	*Zygosaccharomyces Pastori*	5,000	100		Clear
50	*Zygosaccharomyces priorianus*	5,000	2,000	300	Clear
51–72	Twenty-two cultures of yeast-like fungi from pathogenic throats gave varying amounts of visible growth				

TABLE 3

Showing multiplication in Någeli's Medium

CULTURE NUMBER	NAME OF YEAST	INITIAL COUNT	THREE-DAY COUNT	FIVE-DAY COUNT	SEVEN-DAY COUNT	APPEARANCE AT SEVEN DAYS
1	Burgundy wine yeast	500			700	
2	Champagne yeast	500			15,500,000	
3	*Cryptococcus agregatus*	1,000			7,200	
4	*Cryptococcus glabratus*	550			Spread	
5	*Cryptococcus Ludwigii*	600			13,000,000	
7	*Endomyces albicans*	800			40,000	
8	*Endomyces javanensis*	200,000	4.800,000	1,500,000	750,000	Cloudy§
9	*Monilia tropicalis*	360			50,000	
10	*Mycoderma rugosa*	700			300,000	
11	*Mycoderma vini*	500			180,000	
13	Yeast from oysters—Hunter	1,000			2,500,000	
15	*Parasaccharomyces Ashfordii*	285			1,800	
17	*Pichia farinosus*				240,000	
18	*Pichia membranaefaciens*	200			11,500	
19	Red yeast (Snow)	2,000			400,000	
20	*Saccharomyces albus*	1,000			1,100	
21	*Saccharomyces anomolus*	300			400,000	
22	*Saccharomyces capsulans*	20,000	1,000*	19,000†	500,000‡	Very slight clouding
23	*Saccharomyces carlsbergensis*	30,000	2,000*	5,000†	500,000‡	Cloudy—moderate
24	*Saccharomyces cerevisiae*	150.000	4,500,000	910,000	800,000	Cloudy—with sediment§
25	*Saccharomyces ellipsoideus*	500			6,600	
26	*Saccharomyces hominis*	500			10,000	
27	*Saccharomyces intermedius*	36,000	1,200*	6,000†	1,500‡	Clear
28	*Saccharomyces logos*	200,000	2,000,000	5,000,000	10,000,000	Cloudy§
29	*Saccharomyces mandshuricus*	7,200	2,000*	1,100†	750	
30	*Saccharomyces marxianus*	75,000	8,600*	25,000†	100,000	Very slight clouding

31	*Saccharomyces neoformans*	75,000	12,000*	24,000†	300,000‡	Very slight clouding
32	*Saccharomyces Pastorianus*	500		5,000		
33	*Saccharomyces spec*—Plummer	70,000	11,000*	200,000†	32,000‡	Very slight clouding
34	*Torula colliculosae*	500			12,000	
35	*Torula communis*	200,000	4,500,000	1,000,000	15,000,000	Cloudy
36	*Torula cremoris*—Hammar	500			2,000	
37	*Torula datilla*	15			0	
38	*Torula glutinis*	2,000+			45,000	
39	*Torula humicola*	1,000			150,000	
40	*Torula mucilaginosa*	500			10,000	
41	*Torula rubra*	6,000	2,000*	125†	40‡	Clear
42	*Torula sphaerica*	400			400	
43	*Willia anomala*	1,500			110,000	
44	*Willia belgica*	200,000	1,860,000	3,000,000	1,500,000	Cloudy§
45	*Willia saturnus*	2			5,000	
46	*Zygosaccharomyces bisporus*	1,500			1,000	
47	*Zygosaccharomyces chevalieri*	2,000+			4,300,000	
48	*Zygosaccharomyces mandshuricus*	50			200	
49	*Zygosaccharomyces Pastori*	0			700	
50	*Zygosaccharomyces priorianus*	25,000	8,400*	18,000	16,000‡	Very slight clouding
51–72	Twenty two cultures of yeast-like organisms from sore throats gave very scant growth in Nägeli's Medium					

* Two days.
† Four days.
‡ Six days.
§ Ten days.

GROWTH IN NAGELI'S MEDIUM

In 1879 Nägeli reported three media for studying the nutrition of certain of the lower fungi. That used in this investigation had the following composition: Water, 1000 grams; ammonium tartrate, 10 grams; dipotassium phosphate, 1 gram; magnesium sulfate, 0.2 gram; calcium chloride, 0.12 gram. This medium was sterilized in the autoclave at 15 pounds for ten minutes.

The technic followed with Nägeli's medium was identical with that described above for Fulmer and Nelson's medium. All chemicals were the purest obtainable and every precaution was exercised to prevent the entrance of any extraneous organic matter into the flasks. Counts were not made as frequently as with the two former media.

Nägeli's medium proved to be less satisfactory than either Fulmer and Nelson's Medium F or Fermi's. As would be expected some of the yeasts gave practically no multiplication while others gave excellent indication that the medium was suitable for multiplication.

In table 3 showing the multiplication rate in Nägeli's medium, a number of species gave very slow multiplication. In case of the latter as was done with similar cases in Fermi's medium, 0.5 cc. of yeast water and more viable cells were added to the culture flasks. When this was done *Endomyces javenensis, Torula communis, Willia belgica* and *Zygosaccharomyces priorianus* showed more active multiplication. The cultures of yeast-like organisms from sore throats also gave very scant growth. The flasks remained either clear or showed only the slightest amount of turbidity.

CONCLUSIONS

Various methods have been used for estimating the multiplication and growth of yeasts in synthetic nutrient solutions. The data from these several methods may not be comparable; this may explain some of the controversies which have arisen in this field of investigation. Distinction should be made between methods which measure growth, multiplication, etc. Yeast cells may grow after multiplication has stopped.

The following conclusions are drawn from the data presented in this paper.

1. Fifty pure species of yeasts and twenty-two strains of yeast-like fungi from sore throats were found to multiply abundantly in Fulmer and Nelson's Medium F over a period of eleven months when cultured according to the technic used by these authors.

2. Fulmer and Nelson's Medium F allowed more vigorous multiplication than either of the other two media employed.

3. Multiplication in Fermi's medium was less abundant than in Fulmer and Nelson's Medium F but perhaps a little more abundant than in Nägeli's medium.

4. Nägeli's medium was the least satisfactory of the three media used.

5. Yeasts and yeast-like fungi seem to be able to develop in pure synthetic nutrient solutions if sufficiently heavy inoculations are used. They may develop more rapidly, however, if substances containing "bios" or organic matter are added.

6. Yeast growth and crop are dependent upon a number of factors such as temperature relations of the yeast, acclimatization to a certain medium, purity of the ingredients, chemical constitution of the medium, aeration, hydrogen ion concentration, etc.

7. Each species of yeast probably has its own dietary requirements which are more satisfactorily supplied by one medium than by another.

8. It is unnecessary to assume the requirement of "bios" or any other hypothetical substance to explain the absence of multiplication of a yeast in a synthetic nutrient solution. Lack of multiplication may be due to other things; conversely, the stimulation of growth and multiplication following the addition of a supposedly "bios" containing substance does not indicate that "bios" is necessary since the stimulation might be due to other factors in the "bios" preparate.

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MORPHOLOGICAL AND PHYSIOLOGICAL VARIATIONS IN THE DESCENDANTS OF A SINGLE DIPHTHERIA BACILLUS

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The question of variation in morphology, and in toxin production among the members of the diphtheria group has been of lively interest to the bacteriologist since Roux and Yersin in 1890 made the statement that "they believed the diphtheria group was but a single species" and declared that the morphological and biological differences between the types, (*Bacillus diphtheriae*, Klebs-Loeffler, *B. pseudo-diphthericus*, Hoffman-Wellenhof, and *B. xerosis*, Neisser), were not sufficient to warrant establishing new species.

In reviewing the literature, a striking difference of opinion in regard to the subject of variation on the part of the different authors was very evident.

We find, however, that practically all authors in reporting their experiments remark that they have made use of a *pure* culture, obtained by the isolation of single colonies, by one means or another. In but one instance has it been reported that a single organism was isolated, hence, many of the conclusions given must have been based on the inference that the colonies studied were the progeny of a single organism. Ørskov (1922) said, and we believe correctly, "that by a pure culture we understand, as is well known, a culture consisting of individuals of which we know with certainty that all are descended from one single cell, and one only."

While it is true that all our methods of pure culture study are based on the idea that colonies are developed, each from a single organism, yet in view of the fact that we cannot always

be sure of this, we have felt that any experiments on variation should begin with the isolation of a single cell. Therefore, in our experiments here reported, we have begun with a pure strain of the diphtheria bacillus, isolated from a single cell, and have traced the morphology of the descendants of this single organism and their virulence, and have further confirmed the work by the isolation of other single cells all descendants of the parent strain.

The isolation of a single organism of any species of bacteria is a question of method. The method of isolation should allow of no criticism as to the purity of the strain obtained. Also, in a pleomorphic group such as the diphtheria group it is necessary to know exactly the type of organism isolated. In a series of experiments using methylene-blue as a vital stain good results were obtained in differentiating the various types of the diphtheria bacillus.

The method of isolating a single organism as used by Ørskov, (1922) was found to be suitable to the problem. This method combined with the use of a vital stain was adopted by the writer in obtaining the pure lines used in these experiments.

All morphological types reported were from preparations grown on Loeffler's blood serum slants having a hydrogen ion concentration of 7.4, unless otherwise stated.

A highly virulent culture of the diphtheria bacillus, used for the demonstration of toxin production, was used as a stock culture from which a single organism was obtained by the Ørskov method.

This single organism was an A_1 type of Wesbrook's classification and will be designated hereafter as the parent organism, while all the descendants of this organism will be known as the parent strain.

Observations made on the morphological types of the first six transplants of the parent strain showed, coccoid forms, or the following Wesbrook types: C_2, D_2, A_1, A_2, B_1, C_1. Some forty subsequent transplants of this parent strain showed the types reported above at some time.

All virulence tests made by the subcutaneous inoculation of

a broth culture of the parent strain into 250-gram guinea pigs were positive, with a typical postmortem picture.

Two other single diphtheria cells, both of A_1, were isolated at the same time from the sixth transplant of the parent strain by the Ørskov method. The descendants of one of these organisms will be designated as daughter strain number one, and the descendants of the other organism will be known as daughter strain number two.

The Wesbrook types that developed on the first transplant of daughter strain number one were C_2, D_2, and B_2, while sixty subsequent transplants showed the A_1, B_1, C_1, D_1, C_2 types. Virulence tests carried on from cultures of this series were all positive and comparable to the virulence exhibited by the parent strain and stock culture.

The Wesbrook types as developed in daughter strain number two were in marked contrast to those seen in the parent strain or daughter strain number one. The first transplant showed the D_2, E_2 types while eleven subsequent cultures showed only E_2 types. Ten other transplants showed again the D_2, E_2 types and the remaining thirty-eight cultures gave the CC_2, DD_2, EE_2 of Wesbrook's types.

The virulence of daughter strain number two was as different when compared to the virulence of daughter strain number one and of the parent strain as had been the difference in the morphological types found. In the case of daughter strain number two all virulence tests were negative. The virulence tests were then repeated using increased amounts of culture up to four cubic centimeters with negative results. Then, to be sure that there was no toxin formed, increased amounts of culture of different ages, from one day to two weeks, were used for inoculation with negative results. Finally, inoculations were made of a culture of daughter strain number two which showed the A_1, CC_2, DD_2, EE_2 types of Wesbrook (obtained from experiments reported later in this paper) still with negative results.

In view of the fact that a number of writers have been able to increase the virulence of a slightly toxic diphtheria strain by the addition of amino acids or enzyme preparations it was

thought that under favorable conditions a toxin might be produced from daughter strain number two by some similar method.

Davis and Ferry (1919) were able to increase the potency of the toxin of a diphtheria strain by the addition of cystine to the medium.

Several inoculations into guinea pigs were made with cultures of daughter strain no. 2. The cultures used in the inoculations were of different ages, twenty-four hours to seven days, and grown in media which contained amounts of cystine varying from 0.005 grams to 0.05 grams per cubic centimeter of medium. Cystine however did not aid in the production of a toxin and the results of the inoculations were again negative.

Similar inoculations were made using cultures grown in media containing killed bacterial emulsions, Kent (1922), and water soluble extracts of commercial yeast, potato, and carrot, Kligler, (1919), Kent (1923), these also gave negative results.

An attempt was then made to enhance the virulence of daughter strain number two by contact with the peritoneal fluid of a guinea pig *in vivo.*

Two cubic centimeters of a twenty-four hour broth culture of daughter strain number two were inoculated into the peritoneal cavity of a three hundred gram guinea pig. At the end of twenty-four hours the peritoneal fluid was withdrawn aseptically and transplanted to Loeffler's serum slants. Examination of a preparation made from this culture and stained with Loeffler's methylene-blue showed a pure culture of small coccoid forms which on the subsequent transplant developed a pure culture of C_1 of the Wesbrook types. This culture was designated as P_1.

Four cubic centimeters of a twenty-four hour culture of strain P_1 were inoculated as above and the peritoneal fluid withdrawn as before. Examination of the first transplant showed small coccoid forms with a few of the Wesbrook types. Subsequent transplants showed A_1, CC_1, DD_2, E and E_2 types of Wesbrook. This strain is designated as P_2. A direct smear of the peritoneal fluid showed A_1, C_2, D_2 types of Wesbrook.

Virulence tests, made by subcutaneous inoculation of broth cultures of strains P_1 and P_2 into 250-gram guinea pigs, were negative.

The body fluid as found in the peritoneal cavity of a normal guinea pig had evidently exerted some action upon the diphtheria organism injected, in that the diphtheria organisms had been changed from the granular and barred forms to small coccoid forms through contact with the peritoneal fluid.

From the above experiments it appears that daughter strain number two has failed to produce a toxin but morphologically has produced some of the types found in the parent strain.

Six other single daughter organisms were isolated from the parent strain by the before mentioned method. These daughter strains were subcultured enough times to confirm the previous experiments, in showing that the morphological characteristics of the diphtheria organism were subject to variation. All of the cultures were virulent.

DISCUSSION

The recognition of the Hofmann, or pseudodiphtheria bacillus, as a distinct species from the diphtheria bacillus becomes more, difficult as bacteriological research advances. Until, however overwhelming evidence is presented to differentiate between these two organisms, there will be those who believe there is but one species, of which the Hofmann bacillus is but a variation or mutation.

Lessieur (1901) and Haven (1920) have shown that the morphological types of the diphtheria bacillus are apparently nonspecific. The latter writer has determined two groups of diphtheria by the agglutination test in which there is no evidence of cross-agglutination, and in both groups has found virulent and avirulent diphtheria bacillus. Wesbrook, Wilson, and McDaniel (1899) suggested, and Gorham (1901) noticed, that the barred types were changing to the solid staining types under the influence of body fluids, and believed the Hofmann bacillus to be a morphological variety of the diphtheria bacillus.

Salter (1899), Ohlmacher (1902), and others succeeded in changing the pseudodiphtheria organism into a typical diphtheria bacillus at the same time increasing its virulence by passage through animals.

Goodman (1908) and Meader (1919) were unable to correlate fermentative power and virulence. The former writer obtained a strain of the diphtheria bacillus which produced no acid in broth.

Williams (1902), Berry, and Banzhof (1912), and others have claimed the non-variability of the diphtheria group.

Rickards (1908) has reported variations in virulence among different colonies of the same strain, which work has been confirmed by Baerthlein, Jacobstal, Bernhardt, and Paneth (1913).

Mellon (1917), in his classification of the diphtheroids has considered the Hofmann bacillus as a mutant of the diphtheria bacillus, and concluded by saying "any of the sub-groups may become pathogenic under favorable conditions."

Dixon (1919) says "transmutation differs from variation in degree alone; it is a question of the extent of the modification and the degree of permanence it exhibits."

Walter (1915) in his chapter on mutation has concluded that "to the student of heredity, there are two distinctions of prime importance with respect to mutations. First, that they usually appear full-fledged without preparatory stages, and second, they breed true from the start. Fluctuations on the contrary ordinarily 'revert' to the parental type in subsequent generations."

Morgan (1919) has shown that the "genes," which are the character determiners, stand at definite levels in the chromosomes.

Morgan, Sturtevant, Muller and Bridges (1922) have said that the phenomena known as "non-disjunction" may be the factor which causes variation. It has been shown that when certain genes were absent from the chromosomes, the hereditary characters controlled by those genes were also absent.

"The loss of any character would necessitate the chemical rearrangement of the internal structure of the organism or the intracellular stereochemic recombinations necessary for

the birth of new forms" (Mellon, 1921). The new forms in the case of the diphtheria bacillus would be the non-toxic organisms.

The experiments of Middleton (1915) on *Stylonchia;* Jennings (1916) on *Difflugia;* and Hegner (1918) on *Arcella dentata* have shown mutations to occur in unicellular organisms. The mutations were here due to an unequal division of the nuclear material and environmental agents. It is probable that the same phenomenon has taken place in the development of the non-toxic daughter strain no. 11 of the diphtheria bacillus.

The preceding experiments have shown that there is a distinct morphological fluctuation in the diphtheria bacillus. What is most important, however, a distinctly non-virulent strain of the diphtheria bacillus was obtained by the isolation of a single organism from a virulent parent strain, also descended from a single organism.

A fluctuating variation has been shown by the development of barred Wesbrook types from the solid forms, but a real mutation has been shown by the development of a non-toxic strain.

To explain the mutation from the toxic to the non-toxic diphtheria bacillus, we assume that there is present in the bacillus, organized or unorganized chromatin or nuclear material, which as "chromosomes" divides regularly with the division of the organism and imparts to each daughter bacillus the "hereditary character" of producing toxin.

Therefore, it would follow that as long as all factors were favorable to the regular division of the "chromosomes" and "genes" of the diphtheria bacillus, the bacillus would be toxic.

If, however, the factors should become unfavorable to the organism, the process of "non-disjunction" would take place and there would follow an unequal division of the "chromosomes" or nuclear material, and part of the progeny of the toxic diphtheria bacilli would be non-toxic. The "character" of producing toxin would be permanently lost and the non-toxic strain resulting would be permanently non-toxic.

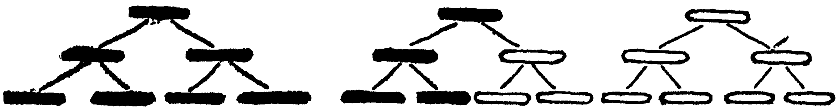
With this in mind, the following diagram has been prepared

to indicate the possibility of mutation from the virulent to the avirulent organism:

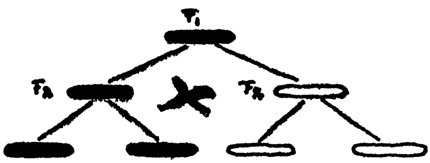
Virulent organism under favorable conditions always throwing virulent offspring

Virulent organism under unfavorable condition throwing both virulent and avirulent offspring

Non-virulent organism always throwing non-virulent offspring



In my own experiments the following diagram would illustrate the actual condition.



F_1 would be comparable to the toxic parent strain, and F_2 black and F_2 white would be comparable to the toxic and non-toxic daughter strains nos. 1 and 2 respectively.

Daughter strain 2 would therefore always be non-toxic, and any increase in virulence of a non-toxic diphtheria bacillus or pseudodiphtheria bacillus, as has been reported must have been according to our experiments, due to a mixed culture.

We feel that we are justified in saying that the non-virulent diphtheria bacillus is a direct mutation of the toxic diphtheria bacillus.

From the above observation we have concluded that:

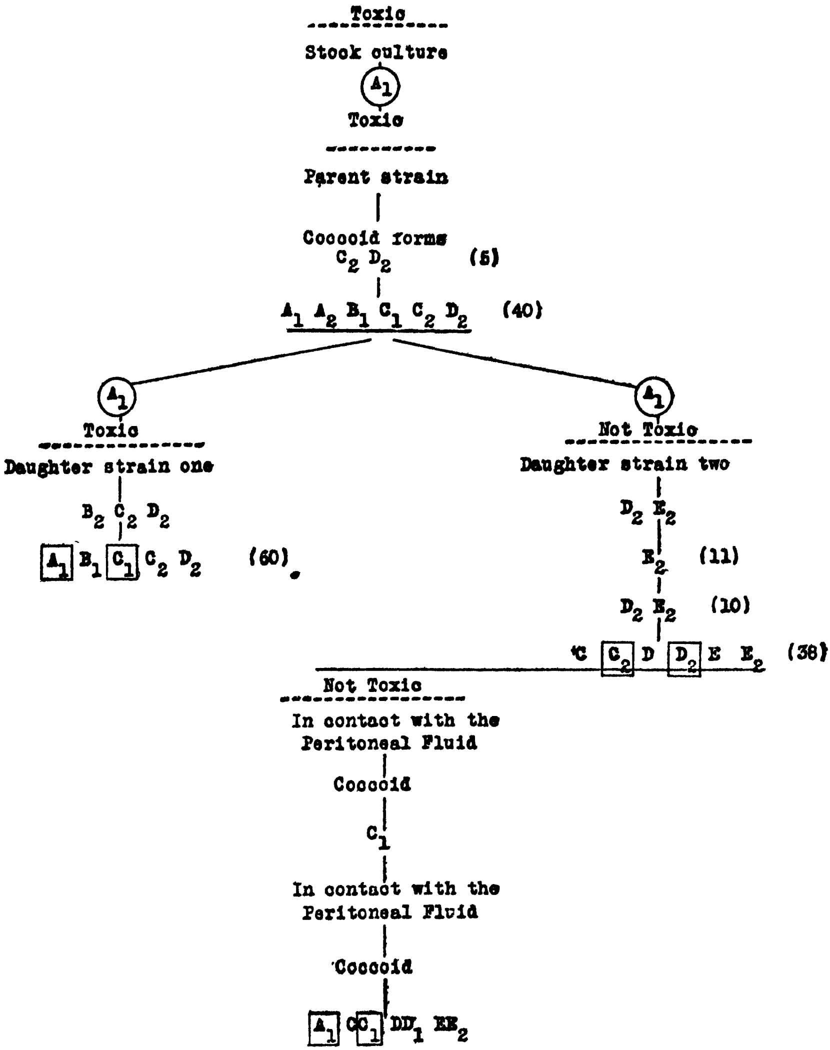
Morphological types of the diphtheria bacillus have no hereditary significance and have no relation to virulence.

The descendants of toxic diphtheria organism may be either toxic or non-toxic.

The non-toxic strains are direct mutations from toxic strains.

The non-toxic strains are permanently non-toxic.

The above conclusions are based on the study of pure line strains obtained by the isolation of a single organism.



The types of organisms and the toxicity of the strains has been indicated in the diagram above. The single organisms isolated are noted by a circle, the numbers indicate the number of transplants made.

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TWENTY-SEVENTH ANNUAL MEETING OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

Madison, Wisconsin, December 29 to 31, 1925

Headquarters, Hotel Loraine

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GENERAL BACTERIOLOGY

1. *Some Fallacious Tendencies in Bacteriologic Taxonomy.* Ivan C. Hall, University of Colorado.

Bacteriology still suffers for the lack of a satisfactory classification and nomenclature. This is thought to be due to the failure of bacteri-

ologists to agree upon suitable criteria in the definitions of taxonomic groups. The use of habitat is particularly criticised as inapplicable to the identification of living organisms and the increased tendency to its use in recent taxonomic efforts is deplored. It is suggested that the attempt to settle taxonomic problems by committee action is likely to prove futile.

A plea is made for definitions of bacterial genera and all larger groupings upon exclusively morphologic (including tinctorial) criteria, reserving physiologic properties for the definitions of species, and excluding serologic reactions (particularly agglutination) from the definition of species.

2. *Serratia indica Bergey, the Red Chromogenic Bacterium Originally isolated by Koch in India.* ROBERT S. BREED AND MARGARET E. BREED, New York Agricultural Experiment Station, Geneva, N. Y.

A discussion of the history and taxonomic relationship of this organism.

This species was originally isolated by Robert Koch in 1884 from the alimentary tract of a Java ape in India, while studying the cholera organism. Cultures were sent to the laboratory in Berlin and have been preserved in various bacteriological laboratories, three strains having been secured for study. The first binomial applied to this species appears to have been *Bacillus indicus* as used by Eisenberg, 1886. Koch used *Micrococcus indicus* in 1887, a name applied to the organism by Crookshank in 1886.

Pasquale, 1891, isolated a red, chromogenic rod from soil in southern Italy which he regarded as probably identical. No other report of its isolation has been found in the literature but cultures of a red chromogen isolated in 1922 at Ithaca, N. Y., by W. A. Whiting from a poorly sterilized milk can have proved to be identical with cultures of the original.

While it is almost identical in cultural characteristics with *Serratia marcescens*, Bizio (*Monas prodigiosa* Ehrenberg), it differs from this well known organism in several distinct characters. In a gelatin stab culture, especially where no peptone or beef extract is added to the medium, it produces a brilliant, orange-red pellicle which readily settles into the liquefied gelatin in old cultures or on being shaken. *S. indica* utilizes urea more readily than *S. marcescens* and gives an abundant, pink, mucous-like growth in a liquid medium containing urea, potassium

chloride, glucose and distilled water. *S. marcescens* grows very poorly in this medium, without chromogenesis. *S. indica* is less variable in morphology than *S. marcescens*. *S. marcescens* under the same condition is highly variable, varying from coccus-like rods to distinct rods and even long filaments. The latter appear particularly well in sugar broths after acid has been produced. Intravenous inoculations of a saline suspension of *S. indica* are reported by previous investigators to kill laboratory animals more quickly than similar suspensions of *S. marcescens*. Tests on rabbits and guinea pigs have confirmed these statements.

Because this organism has been isolated so rarely it may be a rare species. Its wide distribution suggests, however, that it has ordinarily been overlooked or confused with *S. marcescens*, and that it may occur abundantly in suitable habitats.

This investigation has been made possible through a grant given by the American Association for the Advancement of Science.

3. *Fermentation of Citrates. Fermentation of Citric Acid in Various Culture Media.* E. G. HASTINGS, HARRIET MANSFIELD AND GEORGE HELZ, College of Agriculture, University of Wisconsin.

The fermentation of citric acid by bacteria depends on the nature of the medium. Organisms fermenting citric acid in milk do not necessarily ferment it in another organic medium, and those which ferment citric acid in an inorganic medium in which sodium citrate is the only source of carbon do not necessarily ferment it in milk. The nature of the available carbon is probably the determining factor in citric acid fermentation. Organisms found to ferment citric acid in milk are *Escherichia coli, Aerobacter cloacae, S. citrovorus* and *S. paracitrovorus*. Those which do not attack citric acid in milk are *S. lactis, L. casei, L. acidophilus, Aerobacter aerogenes*, casein digesters and lactose fermenting yeasts.

4. *Differential Tests and Their Relation to Habitat of the Coli-aerogenes Group.* S. A. KOSER, University of Illinois, Urbana, Ill.—(Lantern.)

The simpler tests used to separate intestinal *Bact. coli* from other members of the coli-aerogenes group were applied to a series of cultures isolated from known sources representing various grades of intestinal pollution. The tests employed were the methyl red and Voges-Proskauer tests and the test of citrate utilization. Over nine hundred cultures

obtained from the following sources have been subjected to these tests: (1) human and animal feces, (2) pastures, (3) cultivated fields, (4) apparently unpolluted soil, (5) raw sewage, (6) rivers, and (7) springs and other supplies classed as unpolluted. The most important points brought out were that colon organisms resembling those of intestinal origin in respect to the methyl red and Voges-Proskauer tests may be found in apparently unpolluted soil. These types may be differentiated from the true intestinal *Bact. coli* by use of the citrate test; *Bact. coli* from the intestine is citrate negative, the closely related types from soil are usually citrate positive. On the basis of this differentiation, a marked degree of correlation is found between the results of the citrate test and the degree of pollution represented by the various classes of soil and waters examined. In the work thus far, the citrate test has correlated more closely with sanitary conditions than have the other differential tests.

5. Carbon Dioxide Requirements of Bacteria. GEORGE VALLEY AND LEO F. RETTGER, Yale University, New Haven, Conn.

In attempting to answer the question, "Can bacteria initiate growth and develop in the absence of CO_2?" the following organisms (82 in all) have thus far been employed.

Coccaceae: Staph. albus and *aureus* (4 strains), *Strep. pyogenes* (1 strain), *S. lutea* (1) and *S. aurantiaca* (1), and *M. tetragenus* (1 strain).

Spirillaceae: V. cholerae (3), and *V. metchnikovi.*

Bacteriaceae: C. violaceum, E. prodigiosus (2), *Ps. pyocyanea* (4), *Ps. fluorescens* (2), liq. and non-liq., *Zopfius zenkeri, Bact. coli* (12), *Bact. aerogenes* (2 soil and 2 intestinal), *Bact. cloacae, Bact. para-typhosum,* A and B. *Bact. typhi-murium, Bact. abortivo-equinum, Bact. anatum* (2), *Bact. aertrycke* (2), *Bact. pullorum* (4), *Bact. gallinarum, Bact. enteritidis, Bact. typhosum* (11), *Bact. pneumoniae, Bact. dysenteriae* and *L. acidophilus* (7).

Bacillaceae: B. subtilis, B. cereus, B. mesentericus, B. anthracis, B. prausnitzii, B. mycoides (2), and *B. megatherium.*

All of the above organisms have failed to initiate growth in the absence of CO_2.

A study of several clostridium forms is now in progress. The evidence thus far points to the fact that spore-forming anaerobes also require CO_2 for their development.

It appears that CO_2 is necessary for all bacterial development.

In many instances, growth could be prevented by removing the CO_2

from the atmosphere with which they were supplied preliminary to and during the period of incubation. On the other hand, some of the organisms failed to develop only when both the gaseous environment and the medium were made CO_2 free.

In the second phase of the investigation it was shown that, though the bacteria employed require CO_2 for development, different organisms had definite optimum concentration limits, and that 0.3 to 1 per cent CO_2 favored higher plate counts than the amounts ordinarily present in the atmosphere (about 0.03 per cent). No added benefit resulted to the organisms from an increase of the CO_2 content of the enclosed atmosphere to 10 per cent or 20 per cent. Beyond the last-named concentration retardation of growth becomes apparent, due to increased H-ion concentration of the medium.

Different bacterial groups, and often different members of the same genus or species, vary markedly in their carbon dioxide requirements. For example, certain strains of *Proteus vulgaris* are very easily inhibited by CO_2 removal, while others are not; again *B. subtilis* fails to grow in a gaseous environment from which CO_2 is absorbed, while *B. cereus* can be prevented from developing only by the most extreme measures of CO_2 removal from the medium itself.

6. A Rapid Method for the Determination of Sugar in Bacterial Cultures. H. R. Stiles and W. H. Peterson, University of Wisconsin—(Demonstration.)

Instead of determining the titratable acid, hydrogen-ion concentration, or other indirect means of measuring sugar fermentation, it is preferable to analyze the culture for its unfermented sugar. This can be done quickly and accurately by means of the Shaffer-Hartmann micro-method as adapted to bacterial cultures.

Ten cubic centimeters of culture is placed in a 50 cc. volumetric flask, neutralized with NaOH, clarified with 1 cc. of lead subacetate (30 per cent) and without removing the precipitate is deleaded with 3 cc. of disodium phosphate (10 per cent) and the volume made up to 50 cc. The contents are shaken by inverting and in about 3 minutes the precipitate has settled and 2 cc. may be removed without filtration and the sugar determined by the micro-method of Shaffer and Hartmann. The procedure is accurate to within 10 to 20 mgm. per gram of sugar. If a somewhat less degree of accuracy is sufficient, as is usually the case in determining the question of the fermentability of the sugar, 1 cc. of culture is removed with a sterile pipette, neutralized, made to 5 cc. and

the sugar determined without clarification. This modification gives results that are 1 to 2 per cent higher than when the culture is clarified.

7. *A New Method for the Detection of Proteolysis by Bacteria.* W. C. FRAZIER, Bureau of Dairying, Dept. of Agriculture, Washington, D. C.

A new method is described by which small amounts of proteolysis of gelatine can be detected and a rough idea of the amount of this hydrolysis obtained.

Plates are poured of gelatine-agar which contains 0.4 per cent of gelatine together with salts and small amounts of peptone and beef infusion. Duplicate plates are inoculated at the center to form a giant colony and incubated at 30°C. for forty-eight hours. Then one plate is flooded with an acid solution of bichloride of mercury while the other plate is flooded with a 1 per cent solution of tannic acid. In the first a clear zone around the giant colony indicates decomposition of the gelatine as far, at least, as peptones. The width of the clear zone is indicative of the amount of proteolysis. Similarly a heavy precipitate about the giant colony in the plate flooded with tannic acid solution shows decomposition of the gelatine. There is also about the colony of an organism which breaks down gelatine to a considerable extent, a clear zone surrounded by a striking white halo. The tannic acid precipitates the gelatine and decomposition products down through some of the amino acids. By use of the two plates, then, a fair idea can be obtained of the amount of decomposition of the gelatine.

It was also found that by this method the ability of an organism to liquefy gelatine could be determined in two days. This was demonstrated with 215 cultures by comparison with the standard method for determining gelatine liquefication, and by amino acid determinations on a similar solution without agar. Even in cases where the organisms liquefy gelatine without a measurable increase in free amino acid groups, this plate method shows a decomposition of the gelatine.

8. *The Influence of Sodium Chloride on the Colorimetric Determination of pH.* L. B. PARSONS AND W. F. DOUGLAS, Laboratory of the Cudahy Packing Co., Omaha.

Electrometric comparisons have been made between Clark and Lubs buffer solutions and similar buffer solutions 1M, 2M, 3M with sodium chloride, after adding indicator and adjusting to identical colors. From these measurements the sign and magnitude of the salt error was

noted. With the following indicators: thymol blue, cresol red, phenol red, brom thymol blue, brom cresol purple, brom cresol green and brom phenol blue the errors were such that in molar salt solutions 0.2–0.3 of a pH unit (depending on the indicator) must be subtracted from the apparent value to give the actual pH. In 2M salt solutions 0.25 to 0.35, and in 3M, 0.30 to 0.43 pH must be subtracted from the apparent values. These results indicate that a satisfactory correction may be applied to solutions containing salt in the above range of concentrations to yield results which are within the accuracy of the colorimetric method.

9. The Significance of Recent Studies on Meriquinones. W. MANSFIELD CLARK, BARNETT COHEN AND H. D. GIBBS, Hygienic Laboratory, Washington, D. C.

On partial oxidation of benzidine or p-phenylene diamine or their analogues there are formed color compounds consisting of mixtures of the oxidant and reductant. These so-called meriquinones have been very extensively used in various color tests for oxidation processes in biological systems. We have shown that the first stage of oxidation resulting in the characteristic color, is a reversible process and that the complex equilibria involved can be measured by electrometric methods and defined in terms of oxidation-reduction potentials.

It is therefore possible to define numerically the intensity of oxidation required for the production of these color reactions. But it is found that the equilibria are so complex that the amount of color becomes a function of the relative concentrations of total oxidant and reductant, of the hydrion concentration of the solution, of the degree of oxidation at any given total concentration and of the dilution. Furthermore, it has been demonstrated that the condition conducive to the formation of color is also conducive to its rapid destruction.

In consequence of these quantitative findings, it can be predicted that a precise quantitative analysis of the significance of color production in any given case is almost impossible; and that these color reactions will henceforth be relegated to qualitative uses. However, certain gross relationships concerning the degree of oxidation required for color production can be established. Color can be produced by oxidation of members of the phenylene diamine series at potentials comparable to those at which indophenols are partially reduced. Very appreciably higher potentials are required for oxidation of members of the benzidine series. These quantitative relations, when considered in conjunction with the fact that cell suspensions frequently oxidize the phenylene

diamines but seldom the benzidines, supports the conclusion reached by studies made with indophenols; namely that the oxidation-reduction potentials of aerated cell suspensions lie near the centre of the oxidation-reduction scale. This suggests that the cells prefer not only solutions neutral with respect to acid-base equilibria but also those neutral with respect to oxidation-reduction equilibria.

10. Sunlight as a Disinfectant. F. M. Meader, Detroit, Mich.—(Lantern.)

In latitude 42 degrees 15 minutes, at an elevation of about 800 feet above sealevel, on a clear sunny day, direct sunlight will kill *Staphylococcus aureus* in one hour during the middle of the day. The same sunlight, passing through glass 8 mm. thick, will kill *Staphylococcus aureus* in a period of about four and one-half hours. Skylight from a northern exposure will kill *Staphylococcus aureus* in about four hours. Sunlight of the kind mentioned above on March 26, begins to be effective about 9'oclock in the morning, but has very little effect after 3 o'clock in the afternoon.

Wave lengths of sunlight which have bactericidal effect on the organisms studied are confined to the short end of the solar spectrum. When using arc lights as sources of light no bactericidal effect was found in a wave length longer than 310 millimicrons in length, during five hours' exposure. The shortest wave length in solar light at noon of April 1, at above place, was 299.2 millimicrons in length. At 7 o'clock in the evening the shortest wave length was about 310 millimicrons. Since bactericidal properties of sunlight for *Staphylococcus aureus* in direct sunlight are not apparent after 3:30 p.m., it would seem that the effective rays in sunlight must be confined to those rays which are apparent at noon, but not apparent in the late afternoon.

A summary of the theoretical considerations involved in the bactericidal properties of light is briefly reviewed from the literature.

11. Conductivity as Applied to Studies of Bacterial Metabolism. II. Parallelism between Ammonia and Conductivity in Nutrient Gelatine Cultures of Putrefactive Anaerobes. L. B. Parsons and W. S. Sturges, Laboratory of the Cudahy Packing Co., Omaha.

In a previous paper (Abst. of Bact., 1925, ix, 10) conductivity change was shown to be proportional to ammonia change for various strains of *C. sporogenes* and *C. flabelliferum*. An extension of these measurements has been made to gelatine cultures of *C. sporogenes*, *C. bifermentans*, *C.*

histolyticum, C. Reading, C. parasporogenes, C. tertium, and *C. putrificum.* Cultures were incubated in hydrogen at 20°C. and 37°C., determinations being made after two, four, six, ten and sixteen days.

For all cultures a direct proportionality was found between conductivity and ammonia. Change in Sp. Cond. (in reciprocal ohms) $\times$ 1.95$\times 10^4$ = mgm. Ammonia Nitrogen produced per 100 cc. of the medium. The difference between the calculated and observed (Folin) ammonia values was usually less than 5 per cent. The data for *C. histolyticum,* while conforming to the above equation to within 10 per cent are better satisfied by using the constant 1.87 $\times 10^4$. This probably indicates a slightly different type of metabolism by this organism.

12. The Effect of Surface Tension upon the Growth of Lactobacillus acidophilus and Lactobacillus bulgaricus. W. R. Albus and George E. Holm, Bureau of Dairying, Department of Agriculture, Washington, D. C.

L. bulgaricus exhibited no growth when surface tension of the medium employed was depressed with sodium recinoleate to less than 40 dynes, while *L. acidophilus* exhibited good growth in the same medium depressed to 36 dynes. This forms a basis of differentiation for these two organisms and a plausible explanation of the inability to implant *L. bulgaricus* in the lower intestine. Fifteen strains of each organism were employed.

13. Some Relations between the Electrophoretic Potential and Other Characteristics of Variants of Pneumococci. I. S. Falk and M. A. Jacobson, University of Chicago.

Two variant strains of pneumococci obtained by Dr. F. G. Blake, Yale School of Medicine, from a type I culture grown in the presence of homologous anti-serum retain their original characteristics when grown on blood agar. We confirm the finding of early workers that these variants differ from the parent strain in virulence for white mice. Our experiments demonstrate that the electrophoretic potentials on these strains parallel their virulence. The data reported here show that the sequence as well as the absolute magnitudes change when the pneumococcus variants are washed. Our experiments suggest that electrophoretic potential is related in some way to virulence, phagocytability, agglutinability, capsule formation and other characteristics of microorganisms.

A total of 15 strains (single cell) were isolated from Blake and Trask's

A, B, and C cultures of pneumococci (A-type I; B and C are variant strains obtained from A by growth in the presence of homologous antiserum). Experiments with fourteen strains (five from A; five from B; four from C) and with parent strains demonstrate that each of the single-cell strains is indistinguishable from the parent strain from which it was derived with respect to electrophoretic potential, virulence for white mice and serum agglutination.

The isoelectric points of the pneumococcus variants B and C and of A are near pH = 3.0 and are not definitely different. The P.D. for the pneumococcus variants is correlated with the inagglutinability by acid. It is found that the higher the P.D. at pH = 6.5, the lower the pH necessary to cause spontaneous agglutination. The P.D. values for the variant types can be changed relatively by washing them. An inversion of the relative potential on variants A, B, and C is accompanied by an inversion in their agglutinability by acid. Type specific, homologous and heterologous antipneumococcus serum reduces the negative P.D.: the greater effectiveness of the homologous serum in this regard parallels its greater effectiveness in agglutinating the bacteria.

Sodium oleate, which is a specific solvent for pneumococci, dissolves the A, B, and C strains equally well. This finding is contradictory to the usual statement that virulence and solubility (bile) are parallel characteristics. The B and C variants of pneumococci are all of the S type, giving smooth colonies on nutrient or blood agar plates. From each of them we have obtained R strains by growth in the presence of specific anti-pneumococcus serum.

14. The Biologic Origin of Inagglutinability with Freshly Isolated Typhoid Cultures. RALPH R. MELLON, M.D., Highland Hospital, Rochester, New York.—(Lantern.)

It has been found possible to develop inagglutinable typhoid strains from agglutinable ones and vice versa by *in vitro* methods without the employment of immune sera, exudates or other protein substances. This change in type is thought to originate in the reorganization incident to zygospore formation that can be shown with the strains studied. Variants of all degrees of stability have been dissociated out of the plemorphic cycle. Specific metabolic changes may result in similar effects but they are sharply distinguished from genetic alterations by their purely fluctuating character. The physicochemical nature of inagglutinability has been subjected to analysis by cataphoretic methods.

15. Electrophoretic Potentials: The Virulence and Agglutinability of Pneumococci. I. S. FALK AND M. A. JACOBSON, University of Chicago.

The electrophoretic potential bears a significant relation to the stability of a bacterial suspension; and the agglutinability of bacterial suspensions is, in many instances, correlated with virulence for laboratory animals. Studies were made upon the relations between potential and virulence.

From measurements on 76 strains of pneumococci isolated from cases of lobar pneumonia (19 type I; 24 type II; 7 type III; and 26 type IV) it appears that the electrophoretic potentials are different for the several types. The highest potential (circa 11 micra per second) are uniformly found on type III organisms. The most probable sequence of decreasing potential is: III, I, II, IV. The potentials on type III organisms are sharply marked off from the potentials on the others; the potentials on Types I, II and IV organisms are not so sharply marked off. From measurements of virulence of pneumococci for white mice it appears that the sequence of decreasing virulence is: III, I, II, IV and is identical with that for decreasing potential.

Such information as is available indicates that the sequence: III, I, II, IV represents the sequence for the fatality rates in pneumococcus pneumonias of the several types. It was found that for pneumococci of types II, III and IV cultures isolated from fatal cases of acute lobar pneumonia show higher potentials than those obtained from non-fatal cases. The reverse was observed for cultures of type I.

The isoelectric points of pneumococci (types I to IV) are near pH = 3.0 and are not type specific. The electrophoretic potentials are correlated with inagglutinability by acid. The potentials are reduced by specific and by non-specific anti-sera, the greater effectiveness of the former being correlated with the greater effectiveness in causing agglutination.

Sodium oleate, which dissolves pneumococci as specifically as does bile, effects significant increases in electrophoretic potentials. Sodium oleate increases the suspension stability of pneumococci and reduces the titres of serum agglutinations.

16. The Influence of Hydrogen Ion Concentration upon the Viscosity of Bacterial Suspensions. I. S. FALK AND R. W. HARRISON, University of Chicago.

From experiments with *B. coli* it appears that the viscosity, like cer-

tain other properties, of bacterial suspensions is determined by the colloidal nature of bacteria and can be treated as though the bacteria were inert material in the colloidal state. The viscosity of bacterial suspensions is affected by the hydrogen ion concentration. Marked increases in viscosity are produced in zones of pH about the two isoelectric points of the bacteria (pH = 3–4; pH = 13–13.5) where the bacteria show marked acid and alkali agglutination. The effect of hydrogen ion concentration is slight in other pH zones. The viscosity of bacterial suspensions is a function of concentration and is higher the greater the number of bacteria per cubic centimeter of suspension. Our findings indicate that the relation between concentration and viscosity is both qualitatively and quantitatively in accord with the general equation of Arrhenius which describes the relation for colloidal suspensions of inert materials. The viscosity of bacterial suspensions, like colloidal suspensions generally, is a logarithmic function of the relative volume (Φ) occupied by the dispersed particles.

Our findings with bacteria differ from those of Loeb with gelatin and other proteins in that viscosity is *maximal* instead of *minimal* at the isoelectric points. We are led to the conclusion that the increased viscosity which becomes manifest at the isoelectric points of the bacteria is due to an apparent increase in the volume of the dispersed phase which is incidental to the agglutination of the bacteria and the occlusion of water in the clumps of agglutinated particles.

17. The Beginnings of Bacteriology in the Middle West. DR. WM. TREALEASE AND DR. E. A. BIRGE. (By request.)

18. The Rôle of Accessory Food Factors in the Physiology of Microorganisms. C. H. WERKMAN, Iowa State College.

The significance of accessory food factors in the physiology of microorganisms has been studied from three points of view: (1) Accessory food factor requirements of certain yeasts, torulae and bacteria. (2) Stimulative effects of vitamin B on rates of reproduction of microorganisms. (3) Synthesis of vitamins A and B by various microorganisms.

Contrary to general belief and conforming to the view of Fulmer and Nelson, 14 races of *Saccharomyces cervisiae* were found to grow and reproduce continuously in wholly synthetic media employing synthetic methose or succinic acid as the source of energy in Fulmer's synthetic basal medium E. *Saccharomyces ellipsoideus, Torula rosea, Torula*

liquefaciens, Oospora lactis and 12 species of bacteria had reproduced continuously for longer than one year, when discontinued, in wholly synthetic media employing at least three of the four following sources of energy: synthetic methose, succinic acid or distilled glycerol or seven day hot 95 per cent alcohol continuously extracted sucrose.

No addition of bios or vitamin B to the medium is required for the continuous growth and reproduction of the above organisms and if any such substance or substances are required they are metabolized by the organism.

That vitamin B exerts no stimulative effect on the rate of reproduction or the nitrogen fixed by certain microörganisms is apparent when coefficients of the rates of reproduction of microörganisms are determined and due consideration is taken of the nutritive effect of extraneous materials present in vitamin B preparations. This was found true for *Azotobacter chroococcum* and *Rhizobium leguminosarum.*

Synthesis of vitamin A or B was determined by rat experiments, both curative and preventive. Vitamin free and wholly synthetic media were used for the growth of the microörganisms. *Azotobacter chroococcum, Rhizobium leguminosarum* and *Oospora lactis* metabolized a substance apparently vitamin B. No such elaboration of vitamin A by the three organisms tested was found.

19. Yeast as a Substitute for Meat Extract in Culture Media. E. JOHANSON AND JEAN BROADHURST, Teachers College, New York City.

Yeast, as a substitute for meat extract, was tested in liquid and agar media. Ordinary meat extract bouillon was compared with a yeast preparation using the same ingredients (10 grams peptone, 5 grams salt to a liter of water), except that 2 cakes of (Fleischmann) yeast were substitued for the 3 grams of (Liebig) meat extract. Equal inoculations of common organisms were made into these media and the resulting growths were plated at intervals. The counts were much higher in the yeast-agar plates.

The unfiltered yeast was so cloudy that colony counting was difficult. A second yeast medium was prepared by decanting the yeast preparation already described. This yielded a much clearer agar, and the higher counts characteristic of it may be attributed to the greater degree of accuracy in computing the smaller colonies.

Typical plate counts secured from water suspension of common organisms are given in the following order: decanted yeast, unfiltered yeast and meat extract: *Serratia,* 30,600,000, 16,200,000 and 25,500,000;

enteritidis, 24,320,000, 19,840,000 and 19,840,000; *putrificus*, 50,560,000, 35,840,000 and 47,360,000; and *coli*, 198,460,000, 159,360,000 and 155,520,000. In routine examinations of drinking water, swimming pools, and milk the yeast-agar preparations proved quite satisfactory for plate counts, e.g., brook water: decanted, 19,840, meat extract, 14,720; swimming pool: decanted 32,640, meat extract, 20,480; and milk decanted, 8,000, and meat extract 3,000 per cubic centimeter.

For lactose-neutral red determination of coli the yeast preparations gave slightly better results on the whole than meat extract lactose-neutral red.

In some sixty sets of dilutions ranging from 1/100 to 1/10,000, using specific organisms and liquids of sanitary importance, such as water and milk, the yeast-agar counts have averaged $1\frac{1}{4}$ to $1\frac{1}{2}$ times the meat extract counts. The substitution of yeast for meat extract might therefore be accepted for simple bacteriological work, and the addition of yeast might be expected to help in the presumptive tests made to detect organisms of the *coli* group.

20. *Observations of Bacterial Inhibition and the Quantity of Inoculation.* WILBERT S. SLEMMONS, Carnation Milk Products Company, Oconomowac, Wis.

I. Salt tolerance. Experiments were conducted with carefully washed suspensions of *Es. coli*, *M. albus*, and *B. mesentericus*, prepared from pure cultures of these organisms obtained from natural sources. These suspensions were standardized to bacterial amounts of 50; 5,000; 500,000; and 50,000,000 per cubic centimeter and these titres checked by means of the plate counting method. These suspensions were used in one cubic centimeter quantities to inoculate sodium chloride, nutrient bouillon of sodium chloride concentrations from 1 to 12 per cent.

The larger inoculations of the three strains used showed a greater salt tolerance for growth than the smaller inoculations. The widest variation in salt tolerance was between the 50 and 50,000 inoculations of *M. albus*, where the first was inhibited at 4 per cent and the last at 11 per cent NaCl.

Ten trials each were made in culturing the strains of various tolerances in each series into sterile NaCl bouillon of various concentration. This was done with *Es. coli* and *M. albus*. Persistence of ability to tolerate high salt concentration was exhibited by *Es. coli* and *M. albus* strains obtained by artificial selection. This persistence is a variable attribute and seemingly cannot be absolutely predicted as there were a minor number of failures.

II. Hydrogen-ion tolerance. Inoculations of the same range as those used in the above showed no marked differences in hydrogen-ion tolerance in the case of *M. albus* and *B. mesentericus* and no difference whatever in the case of *Es. coli.* The range of pH employed was from 2.4 to 6.0 with intervals of .4 of a pH. Further refinements in technique might show such a relation to exist.

21. A "Bacteriophage" Active Against a Hemolytic Streptococcus. PAUL F. CLARK AND ALICE SCHIEDT CLARK, University of Wisconsin.

A "Phage" has been obtained active against a virulent hemolytic streptococcus. This lytic principle was secured by growing the organism with activated sludge from the Milwaukee Sewage Disposal Plant. It presents all the usual characteristics and is of interest chiefly because of the usual failure to obtain the lytic principle against any streptococcus.

22. The Rôle of the Bacteriophage in Streptococcus Infections. L. O. DUTTON, Methodist Hospital, Memphis, Tenn.

In a detailed study of a large number of *Streptococcus* strains the following conclusions are indicated.

1. A large majority of the strains of *Streptococcus* isolated from the human body are mixed with the bacteriophage.

2. It was found that the bacteriophage is the factor responsible for the occurrence of large numbers of Gram negative individuals in certain cultures of the organisms, for the occurrence of certain large grotesque forms, and for the growth of the organisms in agglutinated masses in fluid cultures.

3. The variation in resistance of the organism to the lytic action of the bacteriophage may account for the variation in virulence of the bacterium, and may account for the occurrence of streptococci in the normal throat (mixed strains), the extreme resistance of certain streptococcus infections (resistant strains), and the occasional occurrence of a spontaneous recovery from a streptococcus septicemia (susceptible strains).

Experimental evidence is given in support of the above conclusion.

23. An Example of Bacterial Mutation. ROY W. PRYER, Department of Health, Detroit, Mich.—(Lantern.)

Sporococcus scarlatinae, described in previous papers from this laboratory, can be changed into a hemolytic staphylococcus by the action

of various serums which possess the power to dissolve *Sporococcus.* It is probable that a filterable stage exists between these two forms. Stabilization of mutated cultures is discussed as well as a brief summary of the properties of the two organisms.

24. Mutation-like Alterations of Certain Pathogenic Fungi. D. J. DAVIS, University of Illinois, College of Medicine.

In certain pathogenic fungi, rather sudden mutation-like alterations appear when such organisms are growing on artificial media. Such changes have been noted especially in *Sporothrica Schenkii,* and in *Penicillium brevicaule.* A description of these changes is given with a discussion of their possible significance.

25. A Genetic Inquiry into the Reason for Three Diverse Morphologic Types of Infecting Fungi in Blastomycosis Hominis. RALPH R. MELLON, M.D., Highland Hospital, Rochester, N. Y.

From a single case of blastomycosis we have isolated two of the three recognized types of parasites believed to be etiologic for the disease. The yeast type comes from the recent lesions, and the intermediate or mycelial type from the older lesions. *In vitro* experiments have shown that pure line cultures of the latter type have repeatedly yielded the mould-like or third type. This change in type has the same genetic mechanism as is operative in changes of bacterial type. An ascospore stage has been observed in the life cycle of these parasites. This so-called "perfect stage" of the mycologist permits for the first time their taxonomic allocation among the endomyces.

26. Crystal Formation in Bacterial Cultures. SARA A. SCUDDER, Evans Memorial, Boston, Mass.

The appearance of crystals, which resemble microscopically those of ammonium-magnesium phosphate, have been observed in connection with bacterial growth in a semi-solid medium. This medium consists of a beef heart hormone base with 0.5 per cent agar, 10 per cent peptone and brom thymol blue indicator, adjusted to pH 7.0. The crystals first appear in the zone of growth and later deeper in the agar. Precipitation of the crystals is enhanced by the presence of peptone and ascitic fluid, and varies with the reaction of the medium, the amount of water of condensation and the species of bacteria. No crystals form in uninoculated medium kept for months in hard glass tubes. The experimental data at hand indicate that the formation of these crystals is

the result of bacterial activity. The bacteria which are associated with the most pronounced crystal formation do not ferment the ordinary carbohydrates and intensify the alkalinity of the medium rapidly; those associated with no crystal formation ferment these carbohydrates and intensify the acidity.

27. Measuring the Growth of Bacteria with the Nephelometer. MARY WOTHERSPOON COLLEY, Madison, Wisconsin.

The nephelometer can be used to measure the degree of turbidity in bacterial cultures. The organisms should be cultured in a colorless liquid medium. Two to 10 cc. are sufficient for examination. After thorough emulsification, and sterilization, in the case of pathogenic forms, the material is ready to be placed in the nephelometer cups. The method offers a speedy and accurate means of comparing turbidity with the personal equation reduced to a minimum.

28. Variation of Temperatures within Bacteriological Incubators. A. R. WARD AND H. A. HARDING, Frederick C. Mathews Company.

The simultaneous variation of temperature in different parts of laboratory incubators was studied by means of standardized thermometers graduated to 1°F. To facilitate accurate readings, each thermometer was inserted through a perforated cork into a test tube of water. Before being placed in the incubator, the test tubes of water were heated to 100°F.

The temperature of nine locations within the incubator chamber were studied, as follows: Left, center and right of two shelves, and similar locations 6 inches above top shelf. Variations of 0.5° to 6.5° Fahrenheit were observed among nine incubators.

AGRICULTURAL AND INDUSTRIAL BACTERIOLOGY

1. An Experimental Study of the Organisms Concerned in the Commercial Production of Acetone and Butyl Alcohol by the Biological Method. ELLIOTT R. WEYER AND LEO F. RETTGER, Yale University.

This organism which is of extreme practical significance has by some been incorrectly called *Granulobacter pectinovorum.* It has also gone by the names "*Bacillus butylicus,*" "*Bacterium butylicum,*" etc. It would seem as if re-baptism is highly desirable. It is a Gram-positive anaerobic organism which produces spores.

In the present investigation isolations were made from corn, barley,

soil and the stomach contents of clams. Attempts to isolate the organism from other cereals and from feces have not been successful.

Well developed and sharply defined surface colonies were obtained on malt extract agar plates incubated in culture jars after complete exhaustion of the contained air. A more practical method, however, is one in which the air is excluded by inverting the bottom of an ordinary Petri dish in a Petri dish lid containing the inoculated malt extract agar.

All strains are carried in 5 per cent corn mash and transferred every two weeks, and until abundant sporulation has taken place. Viability and ability to produce acetone and butyl alcohol in corn mash is greatly enhanced by making transfers from the old cultures after they have been heated at 100° for forty-five seconds. In this way the production of acetone and alcohol was increased in two years 20 per cent or over.

Inoculating the mash with *Staph. aureus* 12 hours previous to the addition of the spore-containing inoculum results in a good growth and rapid and vigorous butyl fermentation without the use of a vacuum pump. The high acidity produced kills off the *Staph. aureus* within a few hours.

Complex nitrogenous substances are necessary. Protein-containing media and ordinary peptone gave good growths.

2. A Group of Non-Spore-Forming Soil Bacteria of Peculiar Morphology. H. J. CONN, N. Y. Agric. Exper. Station, Geneva, N. Y.

It has been observed for some time that of the bacteria in soil growing on gelatin plates about 50 to 80 per cent are non-spore-forming bacteria producing punctiform colonies in gelatin or agar. They have been called by the author "slow growers," or more recently by a more descriptive designation "punctiform-colony-formers." A study of these organisms has shown them to be very diverse both in morphology and physiology.

Special interest has been aroused by one particular subdivision of this group of organisms. The bacteria comprising this smaller group are called by the author "coccus-forming rods." The character by which this group is distinguished is that they appear for one or two days upon a fresh substratum as ordinary small rods, but after that change in morphology and are indistinguishable from micrococci.

3. The Urease Content of Nitrogen Fixing Bacteria. J. ARLINGTON ANDERSON, Biochemical Department, University of Stockholm and the Bacteriology Department, Central Swedish Agricultural Experiment Station.

Stable enzyme preparations of *B. radicicola* and *Azotobacter chroococcum* were made and their urease content quantitatively determined. The values obtained were very small, being of the order of magnitude of preparations from other soil bacteria, excepting urea fermenters. No difference was found between rod and bacteroid forms of *B. radicicola*. The author concludes that while it is not impossible it appears very improbable that urease performs a rôle in nitrogen fixation.

4. The Root-Nodule Organism of Dalea Alopecuroides (Wood's Clover). A. L. WHITING, University of Wisconsin.

The bacteria from the root nodules of *Dalea alopecuroides* have been isolated in pure culture and their cultural characteristics studied. These organisms grow rapidly on mannitol agar, forming a slime which is opaque and resembles somewhat the growth of the nodule bacteria of lupines.

From the results of many cross-inoculation tests, it has been found that this organism does not form nodules on any of the common legumes and hence it has been assigned to a separate group. Because of the general characteristics of the organism, it belongs to the bacillus group of the nodule bacteria, *Bacillus radicicola.*

5. A Possible Explanation for the Specificity of Cross-Inoculation Groups in the Legume Family. I. L. BALDWIN, University of Wisconsin.

The seed protein relationships existing between certain members of the legume family has been investigated by means of serological reactions, using the precipitin and anaphylaxis reactions. The results of this investigation indicate that the legumes belonging to the same cross-inoculation groups possess similar seed proteins. The legumes belonging to different cross-inoculation groups appear to have proteins of decidedly different characteristics.

6. Seasonal Variations in the Ammonia and Nitrate Content of Lake Waters and Their Relation to Certain Types of Bacteria. B. P. DOMOGALLA, Biochemist, City of Madison.

The seasonal variation in the ammonia, nitrate, and oxygen content of certain of the inland lakes of Wisconsin was studied for two and one-half years. Variations in these substances was found to be due almost entirely to a seasonal fluctuation in the bacterial flora of the lakes. Somewhat similar differences were noted in the surface waters, but were more marked in the bottom waters.

The ammonia and nitrate content reach a maxium about March, at a time when the ammonifiers and nitrifiers are most active. During mid-summer and fall nitrate reduction becomes more active than at other seasons of the year and hence the disappearance of nitrates.

7. *Oxidation of Sugar on Trickling Filters.* MAX LEVINE, G. W. BURKE AND J. H. WATKINS, Iowa State College.

A 0.5 per cent skim milk to which various concentrations of lactose were added was passed through an experimental lath trickling filter. The filter was constructed so that samples could be taken at every foot of depth. Reducing sugar was determined gravimetrically.

With an initial content of 321 p.p.m. of lactose, the effluent from the first one foot of the filter contained only a trace. With 1100 p.p.m. of lactose the effluent from the first foot showed 176 p.p.m. and that from the second foot none. Beginning with 3456 p.p.m. of lactose the quantities remaining after various depths of filtration were as follows: 1 foot, 1480; 2 feet, 855; 3 feet, 360; 4 feet, 183; 5 feet, 91; 6 feet none.

Members of the colon group of bacteria are important in the oxidation of acids produced by the initial fermentation of lactose.

8. *Some Observations on the Purification of Creamery Wastes.* MAX LEVINE, G. W. BURKE AND J. H. WATKINS, Iowa State College.

The disposal of creamery wastes has become a vexing question in many states. The commonly employed septic or Imhoff tanks followed by intermittent sand or trickling filters have not proven satisfactory.

Carefully collected samples (composites of fifteen-minute samples taken throughout the day) at a number of representative Iowa creameries, indicated that the concentration of wastes varied from about 1 to 5 per cent skim milk. The oxygen demand of these wastes was 10 to 50 times that of domestic sewage.

Anaerobic treatment of creamery wastes is considered theoretically wrong because of development of acidity which prevents proteolysis, and interferes with nitrification in the filters.

A reaction of pH 5.5 was found to markedly inhibit proteolysis of gelatin and casein, and at pH 5.0 many of the proteolytic bacteria isolated from creamery wastes were killed.

A concentration of 1 to 1.5 per cent skim milk is sufficient to develop these detrimental acid reactions.

The acid producing constituents may be readily removed by oxidation on suitable trickling filters. Concentrations as high as 7 per cent skim milk yielded effluents which did not become acid in anaerobic storage.

Stable effluents were obtained with 1.5 per cent skim milk, after filtration through 6 feet of cinders at a rate of 800,000 gallons per acre per day. Oxygen demands of 600 to 800 p.p.m. were reduced to 15 to 40 p.p.m. Stronger wastes did not yield stable effluents.

9. Acidity in Relation to Bacterial Changes Induced by Freezing. JEAN BROADHURST AND LAURA GILPIN, Teachers College, New York City.

Since frozen foods, such as ice cream, have a wide range in acidity, (commercial and home ice creams examined ranging from pH 7.8 for vanilla ice cream to 3.2 for lemon and orange ices), the differences in acidity might be expected to show some relationship to freezing effects, and throw some light upon the varying results obtained in recent milk and ice cream investigations.

Milk samples with varying initial counts (30,000 to 2,740,000 per cubic centimeter) were halved, and lactic or acetic acid was added to one of each of these paired samples, changing the acidity from pH 7.8 to 6.8, 7.5 to 6.4, 7.2 to 6.0, 7.2 to 4.8, etc. These paired samples were frozen (exposed for 24 hours) in the water-cube pans of an electric refrigerator, bacterial determinations (plated dilutions) being made before and after freezing.

Bacterial determination made *immediately* after adding the acid showed an increase in practically all of the treated samples, due, probably, to the "cutting" effect of the acid upon the bacterial masses. *Following freezing,* however, the acidified samples showed a decided drop in bacterial count.

From these results it is evident that the bacterial content of a given frozen milk product may be materially decreased by increasing its initial acidity.

Another conclusion, which may be of far-reaching importance, seems warranted by the *apparent* increase obtained immediately upon the addition of acid. Standard acidities are probably necessary for securing comparable determinations of the bacterial content of milk, ice cream and perhaps any substances including water, where the developing colonies are counted. For convenience, as well as for the effect upon the bacterial masses, such acidity standards should be slightly beyond the highest acidity ordinarily occurring in a given substance, e.g., more acid than 3.2 for fruit ices or more acid than 5.2 for ice creams.

10. Thermophilic Bacteria and Milk Pasteurization. H. A. HARDING AND A. R. WARD, Frederick C. Mathews Company.

Bacteria capable of growing at high temperatures have long been known but an appreciation of the constant presence of such germs in all milk is not yet widely spread.

Spore bearing rods seem most common but it has not been demonstrated that rods are responsible for the outbreaks in pasteurized milk.

The occurrence of large numbers of thermophilic germs in the output of a given plant does not appear to depend upon any recognized factors. The outbreaks occur irregularly and at all seasons of the year. Presumably such outbreaks are of fairly frequent occurrence in practically all supplies of pasteurized milk.

11. Thermophilic Bacteria from Milk. F. W. TANNER AND H. G. HARDING, University of Illinois.

Thermophilic bacteria, though not numerous, have been demonstrated in every sample of milk obtained after the milk had left the barn. From milk seventy-three cultures of thermophilic bacteria were isolated at 55C.° on plain nutrient agar, pH 6.8. It is probable that thermophiles, which do not grow on this medium, are present in milk. All the cultures were motile, Gram positive, spore-forming rods which grew well at pasteurizing temperatures, 62.5°C. (145°F.). Several of the cultures were strict thermophiles; while others were facultative thermophiles, some even growing at 20°C. Most were facultative anaerobes but many were strict aerobes. Most of the cultures digested starch, produced acid and no gas from glucose and saccharose, and did not produce acid from lactose.

12. Thermotolerant Saccharophilic Organisms as a Cause of "Pin-Point" Colonies in the Bacteriological Analysis of Ice Cream. A. C. FAY, Kansas Experiment Station, Manhattan, Kansas.

In making counts of ice cream, on standard plain agar, large numbers of "pin-point" colonies on the low dilution plates (1:100) were noted, but not in proportionate numbers or none in higher dilutions (1:1000).

Re-plating of samples of sucrose agar revealed the "pin-point" colonies in all dilutions and in proportionate numbers. Furthermore, re-plating on plain agar, containing in the higher dilution plates 0.001 cc. of sterilized ice cream, which supplied the same amount of sugar as was present in the lower dilution, resulted in the growth of the "pin-point" colonies in all dilutions.

With a few exceptions all pure cultures isolated from these "pin-

point" colonies would withstand 3 or more pasteurizations at 145° for thirty minutes without decrease in number. All cultures were thermotolerant but not highly thermophilic since none grew above 45°C.

They were either Gram positive, non-spore-forming short rods resembling *S. lactis* or somewhat longer rods irregular in morphology and in longer chains. Biochemical studies were made.

13. Some Facts About Streptococcus Lactis and the Litmus Milk Reaction. Karl J. Demeter, Suedd. Forschungsanstalt fuer Milchwirtschaft Weihenstephan and Department of Dairy Industry, Cornell University, Ithaca, N. Y.

In the litmus milk reaction the curdling power of *Str. lactis* is influenced by different milks. Skimmilk has been proved to be more easily curdled than whole milk, and milk, whose fat is extracted with ether, much easier than skimmilk. Only such strains as had been in artificial culture longer than nine months showed a quicker souring in whole than in skimmilk. But there are no sure signs that the fat content is responsible for that difference.

The reddening of the white reduced curd proceeds more rapidly, the shorter the time required to curdle. The velocity with which that takes place is more closely correlated with the lack of fat than is the case with the curdling power. There exists great variability also between the different strains, so that the penetration of the O from the air cannot alone be the reason of the oxidation of the leukobasic litmus.

There is also much difference in the curdling time and development of the red curd, if the milk is incubated either at 30° or at 37°C. The curdling power is distinctly greater at blood temperature than at 30°. That fact shows clearly that there seems to be a close relationship of *Str. lactis* with *Str. faecalis*, as is suggested by former authors.

14. The Application of Growth Curves to a Study of the Bacteriology of Cheese. W. R. Albus, Bureau of Dairying, Department of Agriculture, Washington, D. C.

Two factors contributing to the cause of abnormal gassy fermentations in Swiss cheese are the physiological condition of the cells of the gas producing organism, whether aerobic or anaerobic, at the time the cheese is made and the physiological condition of the cells of the organism used to suppress the abnormal fermentations, i.e., *L. bulgaricus*.

By the application of the principles of growth curves it was found possible markedly to influence the ability of the causative organism to

produce abnormal gassy fermentations in Swiss cheese, to increase the efficiency of the bulgaricus starter in its ability to suppress abnormal gassy fermentations in Swiss cheese and to increase the efficiency of the lactic starter in the manufacture of Cheddar cheese.

15. *The Destructive Action of Certain Bacteria on The Virus of Tobacco Mosaic.* MAURICE MULVANIA, College of Agriculture, University of Wisconsin.

Pure cultures of a number of bacteria were grown in ordinary bouillon to which had been added definite amounts of filtrates of juice from mosaiced tobacco plants. The tubes containing the bouillon-filtrate combination were allowed to stand for several days to insure bacterial sterility before those to be tested were introduced.

It was found that some of the bacteria destroyed the infective property of the virus when grown in the above cultures while others did not. There appears to be no characteristic common to those which destroyed the virus which was not at the same time applicable to some of those which were harmless to it. A certain cellulose-fermenting thermophilic organism proved to be very injurious to the virus. Likewise such bacteria as *B. proteus* and *B. aerogenes* almost completely inactivated the infective agent of tobacco mosaic. On the other hand, such bacteria as *Sar. lutea*, *B. anthracoides* and *B. Hartlibii* were slightly, if at all, destructive to the virus.

16. *Notes on Yeasts in Carbonated Beverages.* CHAS. E. MCKELVEY, Introduced by Max Levine, Iowa State College.

In a study of deterioration of carbonated beverages, yeasts were found to be the most frequent causative agents. Of over 1500 samples examined during a period of two years about 85 per cent had spoiled as a result of yeast growth.

Of 132 samples of sugar obtained from 125 bottling plants in 28 states 62 were found to contain yeasts (47 per cent).

Carrot agar, saturated with calcium sulfate, was found to be suitable for the development of yeast spores and the production of large quantities for experimental work.

Yeast spores obtained from carrot calcium sulfate agar were tested as to their resistance to heat in syrups of various concentrations of cane sugar. In general the higher the concentration of sugar the longer was the time required to kill the yeasts under examination.

At 70°C., working with a mixture of the spores of 27 yeasts isolated

from carbonated beverages, inoculated into sterile syrups of different densities, the time required to kill was 24° Baume, two minutes; 27° Baume, two minutes; 30° Baume, three minutes; 33° Baume, five minutes; 36° Baume, five minutes. At 36° Baume the liquid is almost a saturated sucrose syrup at room temperature.

17. The Effect of Sodium Benzoate Upon Certain Yeasts. FRED W. TANNER AND LUELLA B. STRAUCH, University of Illinois.

The data collected under the conditions of these experiments indicate a marked difference in resistance of pure yeasts to sodium benzoate. On glucose agar and in glucose broth even in the presence of 0.4 per cent of sodium benzoate, growth was appreciable. *Torula communis* did not grow and one strain of *Sacch. ellipsoideus* was inhibited after the concentration of the benzoate in the broth had reached 0.15 per cent. Another strain of *Sacch. ellipsoideus* showed a greater resistance; it grew in the presence of even 1 per cent, but the growth was slower and less abundant. The fungi used seemed to be able to tolerate the sodium benzoate better when growing on a solid medium containing it than when growing in a liquid medium. Two of the strains of budding fungi, *Mycoderma vini* and a pure culture from vinegar (No. 18) were able to grow apparently as well in the presence of 1 per cent of sodium benzoate as in the controls.

Sterile apple juice was almost as good a medium for the yeasts as the sterile glucose broth. However, when sodum benzoate was added the growth was markedly inhibited since none of the fungi grew after 0.01 per cent had been added. The same was also true for the amount of alcohol formed.

Attempts were also made to study the preserving effect of sodium benzoate in catsup. No growth of the yeast could be secured even in control tubes containing no sodium benzoate. The most important facts brought out are the marked differences among yeasts in resistance to sodium benzoate; and the great influence exerted by the chemical constitution of the material to be preserved.

18. The Rôle of Starch in The Cracking of Chocolate Creams. BURTON G. PHILBRICK, Skinner, Sherman and Esselen, Inc. Boston, Mass.

Examination of cracked or blown chocolate creams has shown the presence of a facultative anaerobic bacillus, capable of fermenting glucose. The bacillus probably enters through some one of the ingredients but it is difficult to establish which is the infecting material, as the spoilage is often not apparent until the materials have been consumed in the

factory process. On the other hand examination of the moulding starch will generally show the presence of the organism, since the starch acts as a secondary infecting agent long after the original has been eliminated by use. The infected starch should be discarded to effect a complete and final elimination of the infection.

19. Sugar as a Source of Anaerobes Causing Explosion of Chocolate Candies. JOHN WEINZIRL, University of Washington, Seattle, Wash.

Thirty-three samples of sugar of various kinds were analyzed and the relative number of anaerobes present determined. Anaerobes were found in 85 per cent of the samples tested. The different kinds of sugar showed about the same number of anaerobes, but the number of samples of each kind was too few for drawing positive conclusions. Five types of organisms were found, as follows: *Clost. sporogenes, Clost. putrificum, Clost. aerofetidum,* and two not identified.

COMPARATIVE PATHOLOGY AND IMMUNOLOGY

1. Studies on the Etiology of Distemper in Foxes. R. G. GREEN AND H. O. HALVORSON, Medical School, University of Minnesota.

Epidemics have occurred on silver fox ranches entailing a mortality as high as 70 per cent. The most common lesions have been pneumonia, intestinal lesions, and an enlarged spleen, with a general toxic effect on all organs.

In some epidemics pneumonia occurred only occasionally, with the upper respiratory tract normal. The blood, abdominal organs and central nervous system were found to contain the virus. Filtration experiments showed the infective agent to be non-filterable. The most constant bacteriological finding was the presence of members of the *Salmonella* group. They appeared in pure culture in most ranch foxes. They occurred in pure culture almost always when the disease was transmitted by injection of infective material.

Pure cultures of *Salmonella* obtained from foxes were pathogenic and would cause death in from twelve days to thirty days producing the typical disease. After injection the organism can be recovered in pure culture at autopsy.

2. Changes in Intestinal Flora of Rats on a Calcium Deficient Diet. RACHAEL E. HOFFSTADT AND SYLVIA J. JOHNSON, Department of Bacteriology, University of Washington.

Twenty-six hooded and white rats were fed on a diet deficient in calcium and ten on the same diet with calcium supplied. Fecal examinations were made at intervals of seven days for total bacterial count and types of aerobic and anaerobic organisms present. Autopsies at death were performed on all animals. It was found that the calcium deficient diet caused a definite constipation as shown by physical symptoms and a lowered bacterial count. This may or may not have coincided with the appearance of rickets depending upon the initial physical condition of the animals. The types of organisms present were not different in either group of rats. No gross pathological changes were noted, except an apparent thinning of the wall of the small intestine.

3. L. acidophilus in Epilepsy. N. KOPELOFF, M. P. LONERGAN AND P. BEERMAN, Psychiatric Institute, Ward's Island, N. Y.

Owing to the importance of the gastro-intestinal tract in epilepsy, the investigation of *L. acidophilus* in this condition was undertaken. A series of 15 epileptics were carefully observed for one month, following which 12 of these (the other 3 had only one convulsion per month or less) were given one liter of sterile milk for forty-two days. The methods employed here have been described in "Lactobacillus Acidophilus" (The Williams & Wilkins Co., Baltimore, 1926).

Ingestion of sterile milk did not influence the number of convulsions. Neither did the ingestion of 60 grams of kaolin daily for one month. But when the same quantity of *L. acidophilus* milk, one liter, reinforced with 300 grams of lactose, was administered for two months, there was a striking reduction in the number of convulsions in 5 patients. A slight reduction was also noted in 4 other patients. Three patients remained the same and one showed a slight increase in the number of convulsions.

In the two-month period following administration of *L. acidophilus* milk all the patients (with one exception) had an increased number of convulsions. It appears, therefore, that the treatment was not sufficiently prolonged to confer permanent benefit. However, the results are encouraging enough to continue the treatment on a larger series of patients. This is now being done as well as the repetition of the above experiments on the same patients.

4. The Influence of L. Acidophilus on the Colon-Aerogenes Group in the Intestine. NICHOLAS KOPELOFF AND PHILIP BEERMAN, Psychiatric Institute, Ward's Island, N. Y.

L. acidophilus has been shown to reduce the *relative* numbers of the colon-aerogenes group in the intestine, but the question of *actual* reduction is a perplexing one. The present study was carried out on a series of 36 epileptic patients under daily observation. Fecal examinations were made weekly for seven months, comprising the periods before treatment, administration of sterile milk or kaolin, *L. acidophilus* milk, and after treatment. Platings were made on whey agar and the Ayers and Rupp synthetic medium for the enumeration of the colon-aerogenes group. The fecal specimens were dried and proper calculation for moisture made.

The results proved to be extremely variable, since the patients were on the routine hospital diet. In fact it was impossible to arrive at any clear-cut conclusions because of the large experimental error. In general it may be said that in about one-half the instances an actual reduction in numbers of the colon-aerogenes group was obtained, when averages were computed. The problem, therefore, cannot as yet be regarded as solved.

5. *Experimental Bacillus pyocaneus Keratitis.* EDNA JACKSON AND F. W. HARTMAN, Henry Ford Hospital, Detroit, Mich.

The virulent strain of *B. pyocaneus* used in this work was isolated from a group of industrial cases of keratitis.

The bacillus was isolated from only seven of eighteen cases all clinically similar. In some *B. pyocaneus* was isolated along with *Staphylococcus aureus* and only the reproduction of the rapidly spreading digesting lesions in rabbits convinced us that the former was the etiological factor. Injection of the culture between the layers of the cornea was the only method of infection which reproduced the disease.

Since the only effective treatment of the lesions was the destruction with the cautery, it seemed worth while to investigate the possibility of active and passive immunity in the rabbit. Results of the serum treatment of the disease in rabbits will be reported.

6. *Cladothrix Isolated from the Genito-Urinary Tract.* SARA A. SCUDDER AND DAVID L. BELDING, Evans Memorial, Boston, Mass.

Three strains of *Cladothrix,* as yet unindentified have been isolated from the genito-urinary tract. Strain A was obtained from the cervix of a child. Strains B and C were obtained in almost pure culture from the prostatic secretion of patients who had clinical signs of chronic urethritis with no clinical or laboratory evidence of gonorrhea. When

first isolated, all three strains morphologically and culturally resembled long-chained streptococci, but under favorable conditions of growth, they showed a marked pleomorphism, varying in form from streptococcus-like chains to long wavy filaments with transverse constrictions and branching. The filaments, which varied from 0.6 to 1.7 microns in diameter and 2.2 to over 100 microns in length showed definite protoplasmic differentiation when stained by an alkaline modification of Gram's method. Lancet-shaped diplobacilli were present in the young cultures and large globoid bodies were observed frequently in the old cultures. The strains grew well in broth which had a reaction from pH 6.8 to pH 8.0 the tangled threads forming fluffy masses which were either suspended in the fluid or adherent to the sides of the tube. In broth which had a reaction over pH 7.6 the long strands interlaced in a weblike formation and if undisturbed, remained suspended from the meniscus. The three strains were similar in carbohydrate fermentation, but different in the quantity and rapidity of acid production. In contrast to the other two, Strain C had shorter chains, produced greater cloudiness in broth, formed acid more rapidly, and presented minor serologic differences.

7. *A Simple Modification of the Brilliant Green Method of Isolating Bacterium Typhosum from Feces.* MORRIS L. RAKIETEN AND LEO. F. RETTGER, Yale University.

The method is essentially that of Browning, Gilmour and Mackie, and used by Robinson and Rettger in their earlier work. The only real departure is the use of a buffer in the brilliant green peptone solution which constitutes the preliminary enrichment medium. The stock buffer employed was a mixture of 7.5 grams each of KH_2PO_4 and K_2HPO_4 in 155 cc. of distilled water. Of this solution 0.4 cc. was added to 5 cc. peptone solution, or 1 per cent. The buffered brilliant green peptone tubes had a hydrogen-ion concentration of pH 6.5. The amount of buffer used was sufficient to keep the pH below 7.0 and thus prevent the removal of the brilliant green from solution during the incubation period. Platings were made from the enrichment tubes after from eighteen to twenty-four hours incubation at 37°C. on the modified Endo's medium of Robinson and Rettger.

This method was employed on more than 200 samples of feces which were artificially infected with *Bacterium typhosum*, with consistent results. In addition, it was used by the authors in the isolation of *Bacterium typhosum* at the Lenox Hill Hospital, New York, during the

typhoid epidemic in the fall of 1924. In the bacteriological examination of the stools of 14 clinical cases isolations were obtained in 70 per cent of the cases. Seven different brands of brilliant green were employed. Grübler's and one of the American brands were the most satisfactory.

8. *The Use of Glycerin in Brilliant Green Bile for the Isolation of B. Typhosus from Feces.* SOPHIE A. DEHLER AND LEON C. HAVENS, State Board of Health, Montgomery, Alabama.

Brilliant green bile does not inhibit bacterial growth in some specimens of feces, thus making difficult the isolation of *B. typhosus*, if present, in such specimens. It was found that the addition of glycerin prevents, to a greater extent, the growth of all fecal bacteria. Various percentages (10 to 30) of glycerin in bile were tried. Difco bile dissolved in 10 and 15 per cent glycerin in distilled water, with various concentrations of brilliant green, did not cause appreciably greater inhibition than the brilliant green bile without glycerin. Twenty and thirty per cent glycerin caused marked inhibition of the fecal flora. Further experiments showed that 20 per cent glycerin bile containing brilliant green in the concentration of 1:10,000 did not inhibit *B. typhosus*, but prevented overgrowth of fecal organisms. Plates streaked with a loopful of this medium containing feces artificially inoculated with small numbers of typhoid bacilli were rarely overcrowded, and frequently typhoid colonies predominated.

The advantage of this medium is the increased ease and accuracy in examining the plates, due to the marked inhibition of the fecal bacteria and the increase in tne numbers of typhoid bacilli.

9. *Immunological Studies in Tuberculosis. IV. Concerning the Resistance to Infection of Animals Sensitized with Killed Tubercle Bacilli.* S. A. PETROFF AND F. W. STEWART, Research and Clinical Laboratory, Trudeau Sanitarium.

Guinea pigs sensitized with dead tubercle bacilli were rendered skin-sensitive to tuberculin. At the height of the tuberculin reaction they were inoculated with living tubercle bacilli. Such animals showed an increased resistance over normal controls inoculated at the same time and kept under the same conditions.

10. *A New Method for Suspending and Counting Living Tubercle Bacilli.* FREDERIC B. JENNINGS, JR., Research and Clinical Laboratory, Trudeau Sanitarium.

Living organisms are suspended in buffer solution, filtered through paper, and counted in a bacterial counting chamber. Such suspensions are free from clumps, do not agglutinate spontaneously, and the counting is simple and accurate.

11. A Study of the Blood Groups Among the American Indians. CLARA NIGG, Department of Bacteriology, University of Kansas.

A study of 800 American Indians shows an incidence of Group I Blood Grouping of 70 to 72 per cent.

12. Data Bearing on the Question of the Size of the Antibody Molecule and the Amount of Antibody Substance Present in an Immune Serum. F. M. HUNTOON, Research Laboratory, H. K. Mulford Co., Glenolden, Pa.

Calculations based on the total solids and nitrogen content of antibody solutions not only enable the placing of a limit to the amount of antibody substance present but in the case of agglutinins establishes an outside limit of the size of the molecule of 31 millimicrons.

13. Precipitation with Fractions of Syphilitic Serum. PEARL L. KENDRICK AND R. L. KAHN, Department of Health, Lansing, Michigan.

An attempt was made to determine what fraction of syphilitic serum contained the reacting substances responsible for precipitation. The serum fractions were obtained by various degrees of saturation with ammonium sulfate. The precipitin reacting substances were determined quantitatively by means of the Kahn test.

Preliminary experiments indicated that the globulin fraction of syphilitic serum obtained after 33.3 per cent saturation with ammonium sulfate contained less of the precipitin substances than the original serum; the fraction obtained after 40 per cent saturation contained almost the same amount and the 50 per cent fraction, the same amount as the serum.

Comparative determinations of the precipitin substances were then made with the fractions obtained after 50 per cent, 75 per cent, 100 per cent ammonium sulfate saturation and the original serum. It was observed that all these fractions as well as the original serum were of approximately the same potency, indicating that the albumin fraction was free from precipitin substances. This observation was corroborated by negative findings given by the albumin fraction obtained after com-

pletely saturating the supernatant fluid of the 50 per cent globulin fraction.

These findings indicate that the globulin fraction of syphilitic serum obtained after 50 per cent saturation with ammonium sulfate contains all the reacting substances responsible for positive precipitations as determined by the Kahn test.

14. The Effect of Certain Substances on the Precipitin Reaction. CORNELIA M. DOWNS AND KENNETH GOODNER, Dept. of Bacteriology and Immunology, University of Kansas, Lawrence, Kan.

In the precipitin-preciptinogen reaction the presence of foreign protein does not increase the prozone nor inhibit the reaction.

The precipitate dissolves in weak carbonate solutions. Preciptinogen is present in the fluid but no precipitin is detectable until the solution is neutralized. The precipitate is completely dissolved in 40 per cent glucose with the appearance of both precipitin and precipitate in the solution.

15. A Rapid Method for the Macroscopic Agglutination Test. ARLYLE NOBLE, Medical Research Department, Park Davis and Company, Detroit, Mich.

Using concentrated antigen, with small quantities of serum and antigen, shaking is substituted for incubation. Readings are made in from two to twenty minutes.

16. Placental Transmission of Foreign Protein. FRANCES E. HOLFORD, University of Wisconsin.

The placental transmission of foreign protein is under investigation. By precipitin tests on the serum of the new-born, transmission of crystalline egg albumin injected subcutaneously near term has been demonstrated both in normal and in highly immunized rabbits.

17. Experiments on the Solubility of Pneumococci in Bile and in Sodium Oleate Solutions. I. S. FALK AND S. Y. YANG, University of Chicago.

We have found that washed suspensions of pneumococci in distilled water are usually bile soluble. An occasional preparation is refractory to the solvent action of the bile. The chlorides with monovalent cations (Na, K, NH_4, Li) in relatively low concentrations inhibit the dissolution of washed pneumococci by bile; in higher concentrations these chlorides

do not inhibit, indeed, may accelerate the dissolution of the bacteria. Chlorides with divalent cations (Ca, Ba) behave quite differently. They are found to inhibit bile solution of pneumococci more effectively in high than in low concentrations. Of the anion series tested, NaOH and Na_3PO_4 are cytolytic to pneumococci; Na_2HPO_4, NaH_2PO_4, Na_2SO_4 and $NaNO_3$ are not cytolytic. Cytolysis by NaOH and by Na_3PO_4 appears to be a function of the hydroxyl ion concentration. Peptone, gelatin and ovalbumin appear to inhibit cytolysis by bile in the same manner as $CaCl_2$ and $BaCl_2$. The inhibitory action increases with concentration. The differences in the behavior of chlorides of mono- and di-valent cations recorded here appear to be in harmony with the usual findings in general physiology, whether effects upon animals and animal tissues, upoh chlorophyll-bearing plants, bacteria or upon non-living substances are studied.

In the concentrations in which it is active (1.25 per cent and higher) sodium oleate dissolves only pneumococci of those strains which are also dissolved by bile. In effective concentrations, sodium oleate, in aqueous solutions, dissolves washed pneumococci resuspended in distilled water; it does not dissolve pneumococci which have been resuspended in 0.85 per cent NaCl solution or in the supernatant of the culture fluid from which they had been separated by centrifugalization. The bacteriolysis of washed pneumococci by sodium oleate, sodium hydroxide, or by sodium phosphate (tribasic) is as specific as lysis by bile. Suspensions of bile insoluble strains of pneumococci, of strains of *Streptococcus hemolyticus*, *Str. viridans*, *Str. lacticus*, *M. catarrhalis*, *Sarc. lutea*, *Staph. albus* and if *Staph. aureus* are not dissolved by these reagents.

18. Action of Ultra Violet Light upon the Antigenic Property of P. avicida. L. D. Bushnell, Kansas Agricultural Experiment Station, Manhattan, Kansas.

Ultra violet light has a deleterious effect upon the antigenic properties of *P. avicida* as compared to the effect of heat killed cultures.

19. The Fate of Bact. Typhosum and Other Organisms in a Segregated Vein and in the General Circulation of the Normal and Immunized Rabbit. Ralph G. Mills and Gail M. Dack, Department of Hygiene and Bacteriology, University of Chicago, Chicago, Ill.

A marked reduction of the bacterial count followed the introduction of organisms both in the segregated vein and the general circulation. The

rate of reduction differed however. It is suggested than an additional factor is involved in the destruction in the general circulation possibly the phagocytic power of fixed tissue cells.

20. Notes on the Bacteriology of Two Cases of Undulant Fever in Man. CHARLES M. CARPENTER, Department of Pathology and Bacteriology, Cornell University, N. Y. State Veterinary College.

Discussion of the occurrence of *Brucella abortus* in man, with a bacteriological report of two cases of Undulant Fever.

21. Is Bact. abortus (Bang) Pathogenic for Humans? I. FORREST HUDDLESON, Michigan Agricultural Experiment Station, Michigan State College.

The writer reports three cases of recurrent fever in young men during the past year and evidence which incriminates *Bact. abortus* as being the causative agent.

In the first case, no hemo or urine culture was made, but the agglutinin titer of the blood serum was found to be 1:400 one year after the appearance of clinical symptoms. This case resembled the ambulatory type of recurrent fever.

The second case, clinically resembled the typical type of recurrent fever. The blood serum, one week after the appearance of clinical symptoms, showed an agglutinin titer of 1:1000 for both *Bact. abortus* and *Bact. melitensis.* An organism was cultivated from the blood in atmosphere containing 10 per cent CO_2 which resembled *Bact. abortus* culturally and serologically.

The third case resembled the ambulatory type of the disease. The blood serum shows an agglutinin titer of 1:200 and positive culture. All three of the young men have worked with *Bact. abortus* in the laboratory. Case No. 3 has also worked with *Bact. melitensis.*

Cases No. 2 and 3 had been drinking raw milk for some time from the same source. The raw milk contained *Bact. abortus* in large numbers.

22. A Method of Increasing the Virulence of Cultures of Clostridium chauvei by the Use of Ferric Salts. JOSEPH P. SCOTT, Kansas Agricultural Experiment Station, Manhattan, Kansas. (Read by Dr. L. D. Bushnell.)

The addition of ferric sulphate or ferric chlorid to Hall's medium caused a marked increase in the virulence of strains of *Clostridium chauvei* inoculated on such media.

23. Generalized Torula Infection in Man. B. Z. RAPPAPORT AND BERTHA KAPLAN, University of Illinois, College of Medicine.

A study of the thirteenth case of torula infection in man reported in the literature. Clinically a chronic meningitis, diagnosed antemortem by positive blood and spinal fluid cultures. Report of the pathological findings.

24. Influence of B. Welchii Toxin on Erythrocytes in Vivo and in Vitro. GUILFORD B. REED AND J. H. ORR, Queen's University, Kingston, Canada.—(Lantern.)

B. Welchii toxin injected intravenously into rabbits produces rapid destruction of red-blood cells. If the dose of toxin is large the destruction is rapid and may be followed by the death of the animal. If the dose is smaller the destruction is less rapid and the anemia may be transient. In either case, however, the red-cell destruction is accompanied by a profound alteration in the structure of the cells characterized in the first stages of the toxemia by an increase in microcytes followed later by an increase in macrocytes, poikilocytes, polychromatophilia and budding-cells.

Similar changes are observed when *B. Welchii* toxin is mixed with defibrinated blood or with washed erythrocytes suspended in physiological salt solutions.

25. Pernicious Anaemia-like Blood Changes Accompanying B. Welchii Infections in Rabbits. GUILFORD B. REED AND J. H. ORR, Queen's University Kingston, Canada.—(Lantern.)

A virulent *B. Welchii* culture kept for several days in the ice box and injected in suitable numbers into rabbits subcutaneously, intramuscularly or intravenously produces an infection which may terminate fatally in from twelve hours to three weeks or may persist for two to five weeks and may be followed by apparently complete recovery of the animal. Such infections in rabbits are always followed by an anaemia the extent of which is roughly proportional to the activity of the infection.

Where the *B. Welchii* infection is very active there is a marked decrease in the number of circulating erythrocytes, and increase in the color index and a profound alteration in the structure of the red-cells characterized by anisocytosis, poikilocytosis, polychromatophilia and nucleated red-cells. Where the infection is less active there may be no decrease in red-cell count but at the same time a measurable anisocytosis and poikilocytosis.

26. *Hemolytic Streptococci of the Beta Type in Certified Milk.* J. HOWARD BROWN, W. D. FROST AND MYRTLE SHAW, University of Wisconsin.

Over 90 strains of beta hemolytic streptococci were isolated from the cows and mixed milk of a number of certified milk herds including about 900 cows. There was nothing abnormal about the condition of these dairies and the milk is not known to have caused any infection in the consumers. The streptococci were carefully studied in comparison with strains of *Streptococcus epidemicus* from most of the well studied milk-borne epidemics of septic sore throat which have occurred in the United States since 1911. All the strains of *S. epidemicus* were found to be alike in being encapsulated, in producing rapid hemolysis of red blood cells in fluid media, in being pathogenic for mice, in producing a final hydrogen ion concentration not higher than pH 4.8 in glucose broth, in fermenting salicin but not mannite, and in failing to hydrolyze sodium hippurate. All of the bovine strains differed from *S. epidemicus* in one or more of the above characters. There is no evidence that any of them are pathogenic for man. No known single test is sufficient to differentiate the bovine strains from those which may be pathogenic for man.

Hemolytic streptococci, non-pathogenic for man, are to be found in the milk of many clinically normal cows and probably in the mixed milk of all herds of more than a few cows.

The streptococcus of milk-borne septic sore throat is a well defined species or variety which may be distinguished from other hemolytic streptococci found in milk by the tests described. It retains its characters unchanged during years of cultivation in the laboratory.

27. *Studies on the Streptococcus of Epidemic Encephalitis.* ALICE C. EVANS AND WALTER FREEMAN, Hygienic Laboratory, Washington, D. C.

A streptococcus was obtained from the nasal washings during life and from the midbrain and heart's blood taken at necropsy from a human case of epidemic encephalitis. This streptococcus is highly virulent for rabbits when inoculated into the brain, causing death in from five and three-quarters to 18 hours when the inoculating dose is 0.25 cc. of undiluted broth culture. When inoculations were intracerebral the minimal lethal dose was found to be 0.25 cc. of culture diluted 1 to 10,000. Occasionally infection followed heavy intravenous

inoculations. In that case the brain was always found to be heavily seeded with the streptococcus.

Filtrates of an emulsion of the human brain, of the emulsions of rabbits' brains, and filtrates of cultures have given uniformly negative results when inoculated directly into the brains of rabbits. By planting the filtrates in meat medium, however, virulent cultures have been obtained repeatedly, and they have shown that the organism is in a filterable form in the human brain, in the rabbit brain, and in cultures.

The experimental disease produced in rabbits and monkeys closely resembled epidemic encephalitis of man. The symptoms and histological lesions are discussed.

The same streptococcus was also obtained from the nasal washings, of saliva, or both, from all of the three other patients examined, who were suffering with the chronic form of the disease and from the throats of normal persons.

Serologic tests have not yet been made.

The streptococcus here described was identified with the organism obtained from a human case and described by von Wiesner as the cause of epidemic encephalitis. It agrees also with the streptococcus which Rosenow has obtained repeatedly from human cases. Culturally there is no characteristic which would distinguish it from an ordinary *Streptococcus viridans* but morphologically it exhibits a variety of characteristic pleomorphic forms.

28. *Further Studies on the Poliomyelitis Precipitin Reaction.* E. C. Rosenow, Department of Experimental Bacteriology, Mayo Foundation, Rochester, Minnesota.

In this study previous findings are corroborated.

The incidence of a positive precipitin reaction at the time of attack of poliomyelitis was almost 100 per cent whereas the reaction almost always became negative within three weeks after onset of attack. The results in persons having abortive attacks were similar. The incidence of positive reactions in normal contacts was 77 per cent; in normal controls within the epidemic zone, 72 per cent; outside of epidemic zone, 18 per cent; and in isolated family groups at the time of epidemics, was on the average about ten times as high as after the epidemic had disappeared. A positive precipitin test with swabbings from the throat indicates the presence of the characteristic streptococcus as determined by agglutination reactions and the production of flaccid paralysis in animals.

29. *The Precipitin Reaction in the Diagnosis of Scarlet Fever and Allied Hemolytic Streptococcus Infections.* E. C. ROSENOW, Department of Experimental Bacteriology, Mayo Foundation, Rochester, Minnesota.

The precipitin reaction with an immune serum, prepared by the intravenous injection of first, the dead bacteria and the living cultures of the hemolytic streptococcus from scarlet fever, has been found valuable in differentiating ordinary hemolytic streptococcus infection of the throat and infections due to scarlatinal hemolytic streptococci. A close agreement was found between the results of the precipitin reaction and the production of toxin neutralizable by scarlatinal immune serums. Infection of the throat due to scarlatinal hemolytic streptococci was found common at the time of an epidemic in persons who had had attacks of scarlet fever and others in whom the Dick skin test was negative. A close relationship has also been found between virulence and toxin producing power, and scarlatinal and other hemolytic streptococci. Some of the former lost toxin producing power and some of the latter acquired the power to produce toxin neutralizable by scarlatinal antistreptococcus serum.

30. *Toxin Production of the Streptococcus erysipelatis.* KONRAD E. BIRKHAUG, Department of Bacteriology, School of Medicine and Dentistry, University of Rochester, Rochester, N. Y.

The toxins employed in these studies were prepared in Douglas' tryptic medium inoculated with cultures of *Streptococcus erysipelatis*, which were isolated from the erysipelatous lesions of patients ill with erysipelas. Among thirty-four strains grown at 37°C. for periods varying from six to ninety-six hours, the maximum toxin production was obtained in lots incubated for about 48 hours. Each of the 34 strains studied was found to yield uniformly toxic filtrates. A skin test dose of 0.1 cc. of a 1:1000 dilution of erysipelas toxic filtrate produced in the skin of susceptible persons a lesion, similar in nature to that obtained in the Schick and Dick tests, which measured more than 1.5 cm. in diameter. Complete neutralization of one skin test dose of the erysilepas toxin was obtained by mixing it with an equal amount of convalescent erysipelas serum, or with 0.001 cc. of erysipelas antistreptococcic rabbit or donkey sera. Neutralization of the erysilepas streptococcic toxin was not accomplished by Dochez' scarlatinal antistreptococcic serum, nor by normal rabbit or donkey sera. During the acute stages of erysipelas the patient's blood serum and urine contained a toxic substance which was

completely neutralized by convalescent erysipelas serum and which disappeared from the patient's blood serum and urine as soon as twelve hours after the administration intramuscularly of 25 to 100 cc. of erysipelas antistreptococcic rabbit or donkey sera. If the disease persisted unchecked by the serum therapy, the skin reaction remained positive until defervescence and definite regression of the erysipelatous lesion occurred.

Positive skin reactions were obtained by one skin test dose of erysipelas streptococcic toxin in 27 per cent of apparently normal adults and in 21 per cent of normal school children. Among 19 persons with definite histories of single and recurrent attacks of erysipelas, 4 persons gave positive reactions with one skin test dose of the erysipelas streptococcic toxin. These findings add further evidence to our previous reports that a specific relationship exists between *Streptococcus erysipelatis* and erysipelas.

31. Studies on Scarlet Fever Toxin. PAUL F. ORR, AND M. S. MARSHALL, State Department of Health, Lansing, Mich.

Scarlet fever toxin was produced in accordance with the procedures of the Doctors Dick from a selected group of strains of hemolytic streptococci isolated either from the throats of scarlet fever patients, or from postscarlatinal discharging ears.

Twenty or thirty minutes exposure of the toxin at 100°C. usually rendered it atoxic; however, at least 45 minutes is necessary to insure 100 per cent negative controls in the skin test. Culture medium, treated as in the preparation of toxin, except for the inoculum, gave no reaction in any skin tests in which it was used as a control. It is not considered as good a control as heated toxin.

The age grouping of individuals susceptible to scarlet fever as evidenced by a positive Dick test showed a maximum susceptibility at from two to four years of age. However, the number of tests on children under three years was relatively small, and it is possible that the maximum susceptibility may occur in a younger age group. An average of 34 per cent of positive Dick reactions was obtained in individuals of all age groups in State institutions.

Very little difficulty was experienced in reading skin tests on colored children.

Some work has been done by the Department on the dosage of toxin necessary for immunization, demonstrating the necessity for a large number of skin test doses for injection subsequent to the first.

32. *Immunization Against Diphtheria and Scarlet Fever With Toxins Detoxified With Sodium Ricinoleate.* W. P. LARSON, Department of Bacteriology and Immunology, University of Minnesota.

Mechanism of detoxification. Quantitative relationship between toxin and soap. Effect of concentration of both toxin and soap. The effect of dilution upon a neutral soap toxin mixture. Results of animal experimentation. Results of active immunization against diphtheria and scarlet fever. The possibility of suppressing epidemics of scarlet fever by active immunization.

33. *The Bacteriology of Chronic Prostatitis and the Localization in Animals of Organisms Isolated.* A. C. NICKEL, Department of Experimental Bacteriology, Mayo Foundation, Rochester, Minnesota.

This paper takes up first, the bacteriology of chronic prostatitis as reported by various investigators, and then compares those bacteriological findings with the results obtained by the author. A further study is made of the relation of chronic prostatitis to systemic conditions and to other foci of infection, and the causal relation of the bacteria to these conditions as found by experimentation, is based upon the elective localizing power of the microbe in inoculated animals.

34. *The Distribution of Fusiform Bacilli and Spirochetes in Tissue.* I. PILOT, University of Illinois, College of Medicine.

In pathological lesions the fusiform bacilli and spirochetes have often a characteristic distribution. The bacilli lie in the necrotic portion while the spirochetes are seen advancing from the margins into the surrounding tissues.

A STUDY OF BACILLUS ACIDOPHILUS FROM THE DIGESTIVE TRACT OF CALVES

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In investigations (Smith and Orcutt, 1925) carried on upon the early diseases of young calves, notably an early diarrhea known as scours the regular presence of certain Gram-positive bacilli and cocci in the normal digestive tract made it appear desirable to study these forms in detail. Two forms were manifestly predominant according to films from various levels of the digestive tract although other species, notably sporebearing anaerobes, were present in small numbers. The present article deals with the dominant Gram-positive bacillus, *Bacillus acidophilus;* the following paper with a second common form, designated as the enterococcus.

The literature contains only a few references to acidophilus bacilli from calves. Küthe (1915) made a study of bacteria in the intestinal tract using material from the stomach, duodenum jejunum, ileum, cecum, colon, and rectum. He noted the appearance and reaction of the contents, made direct microscopic examination of stained smears, and cultured the material aerobically and anaerobically using plain agar plates, acetic acid bouillon, and deep layers of sugar agar. He always found a bacillus which he called *Bacillus acidophilus-polymorphus.* He noted the bacilli in the smears from the contents, usually of all levels, and he cultivated them in good numbers in the acid bouillon while the development in the non-acid cultures was more irregular and uncertain.

The present work was done with material from segments of the small intestine from the duodenum to the ileocecal valves and

usually with material from the fourth stomach and sometimes from the cecum. The calves represent two groups, namely, 8 normal calves, from one to two days up to two to three months of age; and 14 calves having diarrhea, from one to eight days old. These calves had received a milk diet. Among all animals of both groups Gram-positive rods appeared in varying numbers throughout the segments. Usually they were very numerous in the fourth stomach and the predominating type in that region. In the normal cases they were the predominating type throughout the small intestine, or they occurred in more or less equal numbers with Gram-positive cocci. In cases with scours a colon type of organism predominated in the lower segments (Smith and Orcutt, 1925).

In the beginning of the work the material was cultured on standard agar plus horse blood and only a few acidophilus colonies developed on this medium. The reason for the small numbers was probably due to the use of a too alkaline medium. The necessity of a certain degree of acidity for the cultivation of these forms was shown by the results obtained by varying the reaction of the medium, in 2 cases in which according to the films *Bacillus acidophilus* was present in large numbers.

These results indicated that the *B. acidophilus* types were living throughout the small intestine and that ordinary standard agar adjusted to a suitable acidity (pH 6.8) plus horse blood furnished a good medium for the isolation of this organism. Although pH 6.8 has proved a favorable H-ion concentration for an abundant growth, if the acidophilus bacilli were mixed with colon types the agar could be adjusted to pH 5.0, which is still favorable to *B. acidophilus* but harmful to *B. coli*.

The question arose as to whether the acidophilus bacilli actually multiplied in the intestine or were only carried down mechanically from the fourth stomach. To answer this three normal calves were examined carefully by plating measured amounts of material from the fourth stomach and the segments of the small intestine and counting the colonies which developed. All cases gave similar results, two of which are tabulated.

The figures show that in the segments just below the stomach

B. acidophilus occurs in smaller numbers than in the fourth stomach. However, they also show that the acidophilus bacilli are present in large numbers throughout the small intestine and that in every case they exist in greater numbers in the lower segments than in the duodenum. These results suggest that *B. acidophilus* multiplies in the small intestine. The amount of increase varied in the different segments of the cases observed,

TABLE 1*

CALF	REGION	OCCURRENCE (IN FILMS)	CULTIVATION (STANDARD AGAR PLUS BLOOD)
			pH 7.4–7.8
1065	Fourth stomach	++++	++++
	Duodenum	+++	+
	Second segment	+++	0
	Third segment	++	0
	Fourth segment	+++	+
	Fifth segment	+++	0
	Sixth segment	+++	0
	Ileum	++++	+
	Cecum	++++	+
			pH 6.8
1106	Fourth stomach	++++	++++
	Duodenum	+++	+++
	Second segment	+++	+++
	Third segment	+++	++++
	Fourth segment	+++	+++
	Ileum	++	+++

* ++++ indicates large numbers; +++, fair numbers; ++, moderate numbers; +, a few; 0, not cultured.

probably depending on conditions existing in the different regions at different times. Also the multiplication in the intestine is not as abundant as in the fourth stomach although a certain amount does occur.

This group of organisms was first isolated and named by Mo. o (1900). All the earlier reports describe the strains as producing acid but no gas from carbohydrates. Torrey and Rahe (1915) described a gas-producing type which they named *B. acidophil-aerogenes*. They isolated this gas-producing type from the feces of man, sheep, and hens. They described two types of colonies

appearing on glucose oleate agar plates, and also noted that in sugar bouillon the gas types were inclined to grow in particles adhering to the sides and bottom of the tube and tended to produce more acid than the non-gas types. The amount of gas produced varied with different strains and also from time to time with the same strain. The maximum gas production reported was 60 per cent and the minimum 2 per cent. They stated that the gas was chiefly hydrogen and that the ratio of H to CO_2 was 4:1 or 6:1. Later Rahe (1918) presented a classification of aciduric bacteria. He named four groups: *B. bulgaricus*, *B. bifidus*, *B. acidophilus*, and *B. acidophilaerogenes*. These were

TABLE 2

CALF	REGION	NUMBER OF B ACIDOPHILUS PER CUBIC CENTIMETER
1106	Fourth stomach	1,330,000
	Duodenum	261,000
	Second segment	859,000
	Third segment	4,000,000
	Ileum	700,000
1112	Fourth stomach	7,820,000
	Duodenum	930,000
	Second segment	1,642,000
	Third segment	1,865,000
	Fourth segment	5,224,000
	Ileum	690,000

subdivided into classes according to the fermentation of various sugars. The acidophil-aerogenes type was the gas-producing group and *B. bulgaricus* was separated from the others by its failure to ferment maltose.

Recently Cannon (1924) reported a biologic study of aciduric bacteria. He referred to the three main groups described in the literature, namely, *B. acidophilus* (Moro), *B. bifidus* (Tissier) and *B. acidophil-aerogenes* (Torrey and Rahe). Cannon worked with 64 strains of *B. acidophilus* and 34 strains of *B. acidophil-aerogenes* isolated chiefly from sputum and feces of normal human adults. None of his strains were obtained from calves. He described the bacilli as facultative anaerobes but growing well

under aerobic conditions. They did not liquefy gelatin. Some strains coagulated milk and some did not. In his study on the effect of the hydrogen-ion concentration he reported that a pH between 6.0 and 7.0 was most favorable but the majority of cultures grew on media adjusted from pH 4.8 to 7.6.

Küthe's paper (1915) is the only report describing acidophilus types from calves. The chief form which he found he called *B. acidophilus-polymorphus*. This organism he described as a Gram-positive rod with rounded ends often occurring in long chains and sometimes in single cells. These shorter single forms were sometimes slightly motile. On agar it formed dewdrop-like colonies which, when magnified, appeared like anthrax colonies. It also grew in a deep layer of glucose agar appearing in twenty-four hours as small dots, and when magnified the colonies showed smooth or slightly notched edges. In a stained preparation from an anaerobic colony the bacilli were thick and occurred singly and in pairs; sometimes there was a long thick rod with a short form above it like a head, also some comma forms and swollen rods. When transplanted back again under aerobic conditions the colonies were of the grayish dewdrop appearance and the bacilli grew again in chains, but the elements were thicker and more irregular than in the first aerobic culture. The alternate cultivation from anaerobic to aerobic conditions could be continued through a long series. Küthe considered *B. acidophilus-polymorphus* to be a single organism characterized by great pleomorphism and variation from aerobic to anaerobic growth. He further described this bacillus as coagulating milk and reddening litmus whey in twenty-four hours. It did not grow on gelatin and its development on serum was doubtful. It produced no indol and formed no gas. Küthe also mentioned two other rarer acidophilus types. These developed anaerobically and one type failed to grow in aerobic cultures.

The present report on calf acidophilus types includes studies of 17 strains recently isolated and 3 strains which were isolated from calves in 1917–1919 by Marian S. Taylor and have been kept in the collection of stock cultures here since that time. The three earlier strains and 12 of the recent strains belong to the class

designated by Rahe, and later referred to by Cannon as B. acidophilus; and 5 strains, because of the production of gas, fall in the group called *B. acidophil-aerogenes.* In the *B. acidophilus* group 5 strains were isolated from the 4th stomach, one from the 4th segment, and one from the ileum, all from different calves; and 5 strains came from the duodenum of three different calves. In the *B. acidophilaerogenes* group 2 strains came from the 4th stomach and 3 from the ileum.

All strains have certain morphological characters in common. They are all strongly Gram-positive rods, rather broad, and with blunt or square ends, showing considerable variation in length of elements. Some rods are short, some long, and some occur in still longer filaments. The rods are single, paired, or in short or long chains, sometimes twisted and tangled. Certain differences occur among the strains. Some always appear as rather short forms usually in pairs, short chains, and groups, and often in parallel arrangement but with a certain variation in the length of the rods. Other strains show many long forms and develop long chains. These rods are usually straight and broad but the longer chains are often twisted or tangled. The gas-producing strains show the greatest variation. They are likely to show more curved rods and the chains are more tangled.

The growth of these strains has been observed in various media and the fermentation reactions of certain carbohydrates recorded In the first place on the blood agar plates, which were used for the isolation of the cultures, all strains, both gas and non-gas types, showed a similar appearance. In twenty-four hours very tiny colonies with tiny greenish zones developed. Some colonies were only visible with the hand lens. In forty-eight hours the colonies were still small and showed small zones of greenish discoloration. The deep colonies often appeared irregular or jagged. The form of the surface colonies was seen better on the plain agar plates. From the different strains two or possibly three types of surface colony could be distinguished. All colonies were small delicate growths, usually not over 1 mm. in diameter. A few strains developed round, even, rather opaque colonies; other strains formed thin, delicate, irregular colonies; and the

remaining strains developed small, delicate, more or less round colonies, but when examined with a hand lens threads were seen projecting from the edges. The round and irregular colonies occur in both gas and non-gas producing types. In the same strain some colonies may be more jagged than others.

In standard bouillon (pH 6.8) the strains usually produce a small amount of fine sediment and a clouding made up of very fine suspended particles. In sugar bouillon the clouding is usually heavier and particles sometimes adhere to the sides of the tube. This is always the case with the gas strains. Certain strains do not cloud the bouillon but grow as a soft sediment made up of small particles. The strains which produced the sediment in bouillon also developed round colonies, but all strains forming round colonies did not grow as a sediment in bouillon.

All these acidophilus strains were grown in tubes of glucose agar 12 cm. deep. The cultures were inoculated when the agar was still soft after boiling. The medium was quickly solidified without shaking and incubated. The growth occurred throughout the depth of the agar. The colonies developed equally well from the top to the bottom of the tube, suggesting capacity for anaerobic growth.

An experiment with milk cultures was made by inoculating a series of milk tubes with different amounts of a bouillon culture and determining the pH values and the time when coagulation appeared. The amounts of culture used for inoculation were 0.01, 0.05, 0.1, 0.2, and 0.3 cc. The results showed that the tube inoculated with the largest amount of culture was the first and the one inoculated with the smallest amount the last to coagulate. The first sign of coagulation occurred in twenty-four hours in the tube receiving 0.3 cc. No coagulation was visible in the tube inoculated with 0.01 cc. until the 11th day. The coagulation always appeared first at the bottom of the tube. The original pH value of the milk was 6.7, and the H-ion concentration gradually increased. The greater the original inoculation the more rapidly the acidity increased. No visible coagulation occurred until a pH of about 5.8 was reached. A firm coagulum throughout the tube occurred at about pH 5.5 and the

acidity might continue to increase gradually up to pH 4.8. The rate of coagulation depended upon the amount of culture inoculated. The greater the number of bacilli added the more rapid the coagulation; but regardless of the amount of the inoculation, no coagulation was visible until a rather definite pH value was reached, and the coagulation was not complete until a second definite H-ion concentration was attained. In other words, the time factor of coagulation varied with the amount of culture transferred, but the relation between the pH values and the degree of curdling was constant, the curdling always appearing at a rather definite H-ion concentration. On the other hand, the final pH value attained was also variable according to the amount of material inoculated. All tubes inoculated with amounts of 0.05 cc. and below reached a final pH of 5.4 to 5.3 after three weeks, whereas the tubes inoculated with 0.1 cc. or more reached within the same period a final pH of 4.8. A similar test was carried out with additional strains to see if this difference in final H-ion concentration was general. Seven strains were used and the amounts inoculated were 0.2 and 0.02 cc. of a bouillon culture. In every case the same conditions occurred as in the first experiment; namely, no visible coagulation until the pH value was about 5.8; the coagulation was not complete until the pH value was about 5.5; and the final H-ion concentration after three weeks gave a pH of 5.3 to 5.1 for the tubes inoculated with 0.02 cc., and a pH of 4.8 to 4.6 for the tubes inoculated with 0.2 cc.

All the cultures grew in gelatin at 37°C., and after a month no liquefaction occurred. The gelatin solidified as soon as it cooled.

Some experiments were made to determine the effect of the hydrogen-ion concentration on the growth of these cultures. The limiting H-ion concentrations were determined and also the range giving the best growth. The procedure used was to prepare a series of tubes of bouillon with a pH range from 8.0 to 4.4. Standard bouillon was used and N/20 NaOH added to obtain pH values above 7.6, and acetic acid added for the pH values below 7.6. Each tube contained the same amount of medium and was inoculated with the same amount of a forty-eight hour agar growth suspended in sterile distilled water. The growth

was measured by plating and counting the colonies and observing the clouding of the bouillon. The medium used for plating was standard agar pH 6.8 plus horse blood. The colonies were counted after 72 hours incubation. This experiment was made with five strains: two gas-producing strains, 1071 and 1106 (fourth stomach); and three non-gas types, 1078, 1106 (ileum), and 133. Strain 1078 represented the cultures forming more or less even colonies and producing a clouding in bouillon; strain 1106 (ileum) represented the cultures forming round colonies and growing as a sediment in bouillon; and, finally, strain 133 was used as a representative of those which had been under cultivation since 1917. The results for the different pH values, indicated by the appearance of the bouillon and the colony counts, showed that *B. acidophilus* develops over a fairly wide range of H-ion concentrations. The recently isolated strains, including the gas and non-gas types, gave a similar range, but the older strain differed somewhat. In every case there was no growth at pH 8.0 and 4.4. Among the recently isolated strains there was little or no growth at pH 7.7 to 7.8, which indicated that the limiting H-ion concentration on the alkaline side was pH 7.8 to 8.0. Also among the recently isolated strains there was always some growth at pH 4.6, and the gas strains showed a better development than the non-gas types at this H-ion concentration. There was usually a good growth at pH 4.8, but the maximum development occurred between pH 7.0 or 6.8 and pH 5.2. Strain 133 agreed with the other strains in its limiting H-ion concentration on the alkaline side but it had a higher pH value as the acid limit. This strain had been kept on standard agar of about pH 7.4, and this may account for the fact that it was less resistant to acid than the recently isolated strains. Its limiting H-ion concentration on the acid side was pH 5.2 to 5.0. The range for good growth was between pH 7.7 and 5.5. The optimum growth occurred from pH 7.4 to 6.0.

The fermentation reactions of these acidophilus strains were tested on glucose, lactose, sucrose, maltose, and mannitol. Fermented bouillon (pH 6.8) plus 1 per cent of the sugar was used for these tests. One lot of bouillon was used throughout for each

set of sugars. The pH values were obtained by the micro-colorimetric method described by Brown (1924). The per cent of acid produced was also obtained by the usual titration method. All strains produced a certain amount of acid from glucose, lactose, sucrose, and maltose, usually the greatest amount from glucose and the smallest amount from maltose. The gas-producing strains formed as a rule slightly more acid in glucose, lactose, and sucrose, and considerably more in maltose than the non-gas producing types. Only one strain formed a small amount of acid from mannitol. Ten strains were tested in duplicate with 1 and 2 per cent of glucose. After two weeks the results in 7 cases showed practically the same pH values for the two tubes, and in the other 3 cases a slightly greater amount of acid occurred in the tubes containing the 2 per cent of sugar, the difference being the greatest in the gas-producing strains.

In regard to the relative amount of acidity produced the individual strains showed some differences. The titration results were rather more variable than the pH values. Kendall and Haner (1924) in a recent study of the metabolism of *B. acidophilus* refer to three morphologically different types. Two of their types fermented the same sugars and the third type fermented two additional ones, sorbitol and mannitol. In regard to the relative amounts of acidity formed by each type in milk, and in gelatin containing glucose, lactose, and sucrose, they noted certain differences in the quantity of titratable acid produced and also in the rate of acid production. They mentioned that a resultant between the two factors, inherent acidogenesis and cultural adaptability, was the probable explanation of these differences.

Another reaction, which differs from time to time and varies for different cultures of the same strain, is gas production. The acidophilaerogenes strains are quite variable in the amount of gas produced and in the time when gas appears. Thirty or more different records of strain 1071 in fermented bouillon plus 1 per cent glucose in fermentation tubes have been made. Just before these inoculations the strain was plated and a single colony picked in a series of five successive transfers. Each time the

plates appeared pure. An agar slant was inoculated from a colony on the fifth plate and a transfer from this culture was used to inoculate the fermentation tubes. The following examples are mentioned to show the extent of the variations occurring in one strain. Two tubes of approximately the same size and filled to about the same level with one lot of bouillon were inoculated with equal amounts of material from the same culture. In one tube 4 per cent gas was formed in forty-eight hours and a total of 37 per cent appeared in six days; while in the second tube there was only a bubble of gas on the eleventh day and no further increase after fifteen days. A third tube was inoculated from the second and showed no gas after twelve days, although growth occurred as usual; and a fourth tube, inoculated from the third, again contained a considerable amount of gas. In another series, cultures were inoculated from the bulb, neck, and arm of a fermentation tube culture which contained gas. The results showed one culture inoculated from the neck which failed to produce gas, and a second tube inoculated from the same region which contained 34 per cent gas. All the tubes inoculated from the bulb and arm contained gas but in amounts varying from 10 to 31 per cent. Thus even with uniform conditions of the medium and the material for inoculation, the rate and amount of gas production still varied in different tubes. Also cultures containing little or no gas may give rise to cultures producing considerable gas and, *vice versa*, cultures containing gas may give rise to others with little or no gas. Similar variations occurred in two other gas-producing strains tested in shorter series. Torrey and Rahe (1915) also reported variability in the amount of gas produced. However, the calf strains differ from the organisms studied by these workers in the composition of the gas. Torrey and Rahe state that the gas was chiefly hydrogen, but do not mention their method of measuring the gas. In the calf strains the gas, as it is formed in the fermentation tube, is almost totally absorbed by NaOH solution, indicating that it is chiefly CO_2.

An experiment was made by growing *B. acidophilus* and *B. coli* together in mixed culture. Fermented bouillon (pH 7.4) plus 1 per cent glucose was inoculated with a known number of

B. coli and *B. acidophilus*. Pure cultures of each organism were made as controls. The growth and pH values were watched and it was found that *B. coli* grew more rapidly in the beginning, but when the pH value reached 5.2 to 5.0, which occurred after the first twenty-four hours, then *B. coli* ceased to multiply and *B. acidophilus* continued to increase. On the third day the control tube of *B. coli* alone showed the bacilli present in larger numbers than in the mixed culture. The counts gave 2228 M. *B. coli* per cc. in the control, in contrast to 32 M. per cc. in the mixed culture, while *B. acidophilus* was apparently still increasing in the latter. Thus *B. acidophilus* was able to outlive *B. coli* in the acid condition. On the other hand, if no fermentable substance is present, as indicated in a similar experiment of mixed cultures in plain bouillon, then *B. coli* will keep the pH value 7.0 to 7.5. This H-ion concentration is unfavorable for *B. acidophilus* but suitable for the growth of *B. coli*. In this experiment *B. coli* multiplied greatly and continued to live in large numbers for a week, keeping the ascendency over *B. acidophilus*. These results indicate that in mixed cultures *B. acidophilus* is able to check the growth of colon bacilli only when a fermentable substance is present. They are in agreement with Cannon's experiments with mixed cultures of *B. coli* and *B. acidophilus*. Similar experiments with mixed cultures of enterococci and colon bacilli, and also with mixed cultures of the three types, *B. acidophilus*, enterococci, and *B. coli*, are reported in a paper on enterococci from calves.

CONCLUSIONS

1. *B. acidophilus* types are always present throughout the small intestine of young calves and are the predominating forms in the fourth stomach. In normal animals they are the predominating organism in the small intestine or they occur in more or less equal numbers with Gram-positive cocci.

2. *B. acidophilus* multiplies in the small intestine of normal calves but the degree of multiplication is greater in the fourth stomach than in the intestine.

3. Ordinary standard agar of pH 6.8 to 5.0 plus horse blood

proved a suitable medium for the isolation of these organisms. Agar pH 6.8 is the most favorable for abundant growth but the more acid agar is better whenever colon organisms are numerous.

4. All the recently isolated calf strains showed certain characters in common, such as (*a*) similar optimum and limiting H-ion concentrations; (*b*) development of small greenish zones on blood agar; (*c*) growth throughout a layer of agar 12 cm. deep; (*d*) no liquefaction of gelatin; (*e*) coagulation of milk at definite pH values and final H-ion concentrations after three weeks, varying with the amount of material inoculated; (*f*) production of acid from glucose, lactose, sucrose, and maltose. The strains differed in the fermentation of mannitol; one strain produced a small amount of acid from this sugar and all the other strains failed to ferment it.

5. The calf strains may be divided into two groups according to gas production: (*a*) non-gas types, *B. acidophilus;* and (*b*) gas types, *B. acidophilaerogenes.*

6. *Acidophilaerogenes* strains are characterized by variation in the rate and amount of gas production, and the gas is always CO_2.

7. Experiments with *B. acidophilus* and *B. coli* in mixed culture, taken together with certain biological characters, indicate that *B. acidophilus* possesses a greater resistance to acid than *B. coli.*

8. The calf strains correspond in general in biological characters to *B. acidophilus* types isolated from man and other animals by earlier observers.

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A STUDY OF ENTEROCOCCI FROM THE DIGESTIVE TRACT OF CALVES

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A group of organisms described as Gram-positive cocci usually occurring in pairs and commonly found in the intestinal flora has been referred to under various names. Schmitz (1912) mentioned that the German literature contained references to *Micrococcus ovalis* (Escherich) and *Streptococcus lacticus* (Kruse) but seldom referred to enterococci. Thiercelin named this type of organism the enterococcus and hence in French publications this name is generally used.

Only brief references to the enterococcus from calves have been found. Küthe (1915) in his study of the bacteria of the intestinal tract of young calves states that cocci of various forms were frequently present in the intestines. These cocci were Gram-positive and grew well in acid bouillon. Tricoire (1917) in his review of the enterococcus states that enterococci occur in the intestines of cattle and some other animals. He described the organism as a micrococcus intermediate between streptococci and pneumococci, and appearing in animals and young cultures as a diplococcus, sometimes with a capsule or halo. The two cocci might be alike or different in size and form, and joined either in a straight line or at an angle. In older cultures the cocci were grouped in masses or chains and in very old cultures rod-like forms were found.

In the present study the same animals and material were used as described in the paper on *B. acidophilus* in calves (Orcutt, 1926); namely, the 4th stomach and small intestines, and sometimes the cecum, from a group of normal calves of 1 to 2 days up

to 2 to 3 months of age, and similar material from sick calves 1 to 8 days old. Preparations from the intestinal contents stained by the Gram method showed Gram-positive cocci, usually in pairs. The cocci were sometimes slightly elongated and the two elements of a pair sometimes varied in size and shape. A small lighter area might exist around the cocci with a line between the two elements of a pair, suggesting a capsule, but this was not always the case. Also the two cocci in the pair were arranged usually in a straight line but sometimes they were at an angle.

These cocci were present in considerable numbers in the films from practically all intestinal levels of normal calves. They also existed in varying numbers in the intestinal contents of calves suffering from diarrhea. In some cases rather large numbers, and in other cases only a few cocci were seen. The distribution of the cocci as indicated from the films showed that as a rule they were more abundant in the small intestine than in the 4th stomach although a few cases showed the cocci about equally numerous in both regions.

The development of the cocci on plates inoculated from the intestinal contents was variable and sometimes the growth did not correspond to the numbers seen in the films. Also, occasionally from a certain segment the cocci developed in almost pure culture. This indicated that multiplication could occur in the intestine, and the region where abundant growth took place probably depended upon the presence of substances which were favorable to the growth of the cocci.

These Gram-positive diplococci seen in the intestinal contents of the calves have been considered as enterococci. They form one of the groups of organisms always present in the intestinal tract of young calves and their existence there is probably a normal condition. They seem to be only saprophytic organisms, but according to a review by Tricoire they are able at times to produce disease. Tricoire (1917) stated that the enterococcus was a saprophyte of man and animals but that it might become pathogenic. He mentioned that it existed normally in the intestinal tract and was one of the fundamental elements in the intestinal flora together with *B. coli* and *B. bifidus*. He listed various

reported cases of enterococcus infection, including gastro-enteritis, hepatic infections, appendicitis, peritonitis, intestinal intoxications, lung abscess, rheumatism, and also cases of tuberculosis, typhoid and paratyphoid fever with which the enterococcus was associated. Furthermore the organism has been reported in wound infections. Thus numerous cases are on record in which infection is ascribed to an organism designated as enterococcus. In the intestines of the calves used in this study the enterococci occurred in considerable numbers, but there was no disturbance referred to their presence.

Tricoire (1917) described the enterococcus as a facultative aerobe growing at room temperature but with an optimum temperature for growth of 37°C. It developed readily in bouillon and pepton solution, and after 24 to 48 hours it produced a sediment, rising in a spiral without breaking up when shaken. It coagulated milk, did not liquefy gelatin, formed no indol, and generally did not ferment sugars.

Dible (1921) considered the enterococcus of Thiercelin, the *Micrococcus ovalis* of Escherich, and the *Streptococcus fecalis* of Andrewes and Horder as probably belonging to the same group of organisms. He studied a large number of strains isolated from feces and concluded that the enterococci were a rather well defined group which could be differentiated from the streptococci by resistance to heat. He designated a central type and three variants. He described the enterococcus as a lanceolate diplococcus growing in bouillon, sometimes in short chains. Occasionally a slight halo was seen around the cocci, but no capsule was demonstrated. It grew slightly in media alkaline to phenolphthalein and gave no growth in media acid to Congo red. In bouillon it formed a uniform turbidity. It coagulated milk and did not liquefy gelatin. It was insoluble in bile and had no action on red blood corpuscles, producing neither hemolysis nor methemoglobin. The main type fermented lactose, salicin, sucrose, and mannitol. One variant failed to ferment sucrose, a second failed to ferment mannitol, and a third failed to ferment both sucrose and mannitol. None fermented raffinose and inulin. Besides these four groups he also mentioned an occasional variant

capable of fermenting raffinose. He stated that enterococci occurred constantly in stools but were not particularly associated with diarrhea. In a few instances he found the organism in saliva.

Kendall and Haner (1924) studied the metabolism of *Micrococcus ovalis*. They state that Escherich isolated and described this organism in 1886, and that in 1899 Thiercelin rediscovered it and named it enterococcus. Kendall and Haner isolated enterococci frequently from the duodenum of adults. Their cultures represented the four types of Dible and also three additional variants. They considered the fermentation of salicin of importance for identification. They found certain strains which fermented inulin but differed from pneumococci by being insoluble in bile. They considered that the enterococci were normal intestinal microbes of the lactic acid forming type.

The present study comprises twelve cultures from calves. They showed certain differences among themselves. One form agreed with the type organism of Dible. There were three variants, one like Dible's variant I, a second like Kendall and Haner's variant IV, and the third and largest group like variant V of Kendall and Haner.

Their appearance in films from intestinal contents has already been mentioned. In 24 or 48-hour agar cultures all strains gave a similar picture. The cocci were mostly in pairs, scattered or grouped together, and some short chains were also seen. The elements were round or slightly elongated and rather irregular, frequently varying in size. An examination of preparations made with India ink showed no distinct capsules. In plain bouillon the individual strains showed certain differences. In some cultures the cocci were chiefly in pairs and varied in shape, being round, slightly elongated, or irregular. Other strains had a similar form but the cocci were generally smaller, and a third group showed a greater proportion in short chains and the elements more elongated. The India ink preparations from the plain bouillon cultures also showed no definite capsules. In sugar bouillon Strain 156 gave a viscid growth, and when examined with India ink large distinct capsules were visible. None of the

other strains gave this type of growth in sugar bouillon nor did they show definite capsules. In regard to capsule formation, Schmitz (1912) states that the occurrence of capsules was not constant but sometimes they were present and under other conditions absent. Dible, and also Kendall and Haner mention that capsules were usually not demonstrable.

Various cultural reactions of these calf strains have been noted. In plain standard bouillon all the strains produced clouding and seven formed a viscid sediment rising in a spiral when the tube was shaken, and thus corresponding to Tricoire's bouillon growth. The other five strains formed a small amount of fine sediment, mixing easily when shaken. All strains coagulated milk. They all grew well in gelatin at room temperature and caused no liquefaction. The growth on potato was slight. On horse blood agar plates the strains produced deep colonies with small greenish zones and round smooth grayish surface colonies also with small zones of greenish discoloration. These differ from Dible's strains which he stated had no action on red blood corpuscles, but Dible's method for testing the action of the culture on blood was different, which may account for his result.

The fermentation capacity was tested on glucose, lactose, maltose, salicin, raffinose, sucrose, mannitol, and inulin. The result divided the strains into four groups. All strains fermented glucose, lactose, maltose, and salicin. Two cultures failed to ferment sucrose, and these same two and two others did not ferment raffinose but all four gave a positive reaction with mannitol. Seven other strains gave a negative result with mannitol and a positive reaction with raffinose. One strain fermented all the sugars including inulin. As already mentioned, the four groups agree with the type organism and variants I, IV, and V of Kendall and Haner. The majority of the calf strains correspond to their variant V. These groups with the pH values for the calf strains have been tabulated.

These results indicate that the calf enterococci correspond with certain types of the organism isolated from other sources. None of the calf strains in this collection agreed with variants II and III of Dible, or these same types plus variant VI of Kendall

and Haner. The pH values showed a fairly uniform amount of acid produced by the different strains in each sugar whenever fermentation occurred, except Strains 1075, 1082, and 1094, which formed less acid from raffinose than the other cultures which fermented this sugar. Strain 1106 agreed with the inulin-fermenting type of enterococci and differed from pneumococci

TABLE 1

Fermentation reactions of calf strains of enterococci grouped according to the types of Kendall and Haner

GROUPS		FERMENTATION REACTIONS WITH pH VALUES FOR CALF STRAINS							
Kendall and Haner	Calf strains	Glucose	Maltose	Lactose	Salicin	Sucrose	Mannitol	Raffinose	Inulin
Type culture				+	+	+	+	−	−
	1070c	4 8	5.0	5.0	5.0	4.9	5.3	7.6	7.6
	156	4.8	4 9	5.0	5.0	4.8	5.3	7.6	7.7
Var. I				+	+	−	+	−	−
	193	4.9	4.9	5.1	5.0	7.6	5.4	7.5	7.5
	945	4.8	5.0	5.0	5.0	7.6	5.4	7.6	7.6
Var. II				+	+	+	−	−	−
Var. III				+	+	−	−	−	−
Var. IV				+	+	+	+	+	+
	1106	4 8	4.8	5.0	5.0	4.8	5.2	5.0	4.8
Var. V				+	+	+	−	+	−
	1070 (2nd)	4 8	4.9	5.0	5.0	4.9	7.6	4.8	7.5
	1072	4.8	5.0	5.0	4.9	4.9	7.6	4.9	7.5
	1074	4 8	4.9	5.0	4.9	4.8	7.6	4.8	7.5
	1121	4 9	4.9	5 0	4.9	5.0	7.6	4.8	7.5
	1075	4.8	5.0	5.2	5.1	4.9	7.7	5.8	7.6
	1082	4.8	5.0	5.1	5.0	4.8	7.7	5.5	7.6
	1094	4.9	5.0	5.1	5.0	4.9	7.7	5.7	7.6
Var. VI				+	+	+	−	+	±

in being insoluble in bile. All the other strains of enterococci were also insoluble in bile.

In experiments on heat resistance 24-hour bouillon cultures in amounts of 2 cc. were heated in corked tubes in a water bath at 60°C. for 30, 15, and 5 minutes. One cc. of the heated culture

was plated and one cc. added to fresh bouillon. After the cultures had been heated for 30 minutes, six developed clouding in bouillon and showed plates filled with colonies. Another strain resisted heating at 60°C. for 15 minutes, and two others showed a few resistant organisms after 15 minutes exposure in one experiment and none in another; while three strains were consistently sensitive to heat at 60°C. for 15 minutes but withstood 60°C. for 5 minutes. It was noted that the seven strains forming a ropy sediment in bouillon were the most heat resistant. Dible considered heat resistance a character differentiating enterococci from streptococci. He stated that all enterococci in broth cultures survived heat at 60°C. for 15 minutes while streptococci were killed in 5 minutes and that he found only one exception to this rule. According to him, the property of heat resistance was due to the presence of some specially resistant cells since most of those present in the culture were killed.

The limiting and optimum H-ion concentrations were determined by the same method as that described in the paper on *B. acidophilus* except that ordinary standard agar was used for plating instead of agar adjusted to pH 6.8. Three strains were used, 1082, 1106, and 193. The growth after 24 hours changed the initial pH toward the acid side, probably because traces of muscle sugar were present. The organisms were able to grow in an alkaline medium of pH 9.0 and certain strains at a slightly higher pH. No growth occurred in bouillon with a pH value of 10.0. The optimum for all the strains tested was between pH 8.0 and pH 6.0. The acid limit varied with the individual strains. Two cultures multiplied at pH 5.0 but gave no growth at pH 4.8, and one strain failed to grow in bouillon of pH 5.5.

Three sets of mixed culture experiments were made in which the enterococci were grown with *B. coli*, with *B. acidophilus*, and finally with both of these organisms. Plain and glucose bouillon were inoculated with the mixtures and a pure culture of each reserved as a control. The strain of *B. coli* used was one isolated within the year from a case of scours. Colony counts were made to determine the number of organisms inoculated and the subsequent growth occurring in the tubes. In the first

experiment with a mixture of enterococci and *B. coli*, the results showed that in the glucose bouillon both organisms grew abundantly in the first 24 hours. The pH value reached 5.0 on the 2nd day. From the 3rd day on the enterococcus kept the ascendency over *B. coli*. In the plain bouillon tube the two strains multiplied together to about the same degree and at the end of a week both were living in large numbers. The pH at this time was 7.8. Altogether the results indicated that the enterococcus is more resistant to acid than *B. coli*.

Experiments in which enterococci were mixed with *B. acidophilus* were carried out in plain and glucose bouillon of three different pH values,—namely, pH 7.4, 6.8 and 5.5. The mixed growth was controlled by pure cultures of each organism. The development was determined by noting the clouding of the bouillon, examining stained preparations microscopically, and making colony counts. The results showed that in bouillon of pH 7.4 and 6.8 the enterococci always grew very abundantly in the beginning, clouding the tubes in 4 to 5 hours, while *B. acidophilus* showed no visible growth at this time. After 24 hours the acidophilus control tubes were well clouded, indicating that *B. acidophilus* grows well in this medium if it is given time to develop without interference. The mixed culture tubes at this time contained chiefly enterococci. The results were similar in both plain and glucose bouillon. These pH values of 7.4 and 6.8 were within the range of optimum growth for enterococci which gave them the advantage. On the other hand, *B. acidophilus* is generally more resistant to acid than enterococci. In the tubes containing bouillon of pH 5.5 the enterococcus growth was delayed. This H-ion concentration is beyond its range of optimum growth but within that for *B. acidophilus*. In this series of tubes no visible clouding occurred within 4 to 5 hours, but after 24 hours all the cultures were clouded. The mixed culture showed acidophilus bacilli present in numbers equal to or slightly above those of the enterococci. This is in contrast to the development in tubes were the enterococcus multiplication was not delayed. However, after 48 hours the plain bouillon tubes in the pH 5.5 series showed a continued increase of enterococci resulting

in their predominance in the mixed culture; while in the glucose bouillon mixed culture of this series according to stained preparations the enterococci did not predominate but were held in check, probably by the increased acidity. Altogether the results indicate that the enterococcus can prevent the growth of *B. acidophilus* by means of its more rapid multiplication, and that the acid reaction produced in the glucose tube has little effect on the development whenever the early rapid growth of the enterococcus can take place, for the multiplication of *B. acidophilus* was interfered with in both the glucose and plain bouillon tubes of an original pH of 7.4 or 6.8. However, the acid formed in these tubes tends in the end to favor *B. acidophilus* against enterococci, since acidophilus bacilli are slightly more resistant to acid, and in a few cases *B. acidophilus* has been found persisting in such cultures after 3 or 4 days. On the other hand, if the original acidity is beyond the range of optimum growth for the enterococci and within that for *B. acidophilus*, then the enterococcus growth is delayed and *B. acidophilus* has a chance to multiply. Thus two of the factors influencing the growth and final outcome of a mixture of enterococci and *B. acidophilus* are (1) that of the rapid growth of the enterococci, which is favored or interfered with by the original pH value of the medium, but which in itself is harmful to be development of *B. acidophilus*; and (2) the acidity produced in the glucose bouillon, which in the end tends to favor *B. acidophilus* against enterococci.

The third type of mixed culture experiment, in which enterococci were mixed with both *B. coli* and *B. acidophilus*, agreed in its results with the other two groups of experiments. In this case, in the glucose bouillon both enterococci and *B. coli* multiplied greatly in the beginning. The pH reached 5.2 in 24 hours and 5.0 on the 2nd day. *B. coli* was the first organism to disappear and it was survived by the enterococci and *B. acidophilus*. The growth of *B. acidophilus* was checked in the beginning, for it was not recognized in the plate cultures during the first 4 days, but on the 8th day it was growing in the glucose bouillon culture. In the plain bouillon tube *B. acidophilus* was not recognized in plate cultures after the first 24 hours, but *B. coli*

and the enterococci grew well together and at the end of a week both were living in large numbers. The pH of this culture ranged from 7.1 to 7.7. All the mixed culture experiments bring out the effect of the H-ion concentration in relation to the action of these strains against each other. They show that both enterococci and B. acidophilus are antagonistic to *B. coli* by their greater resistance to acid; but enterococci are antagonistic to *B. acidophilus* because of their multiplication in greater numbers, and the acid reaction produced by the two strains in mixed culture in glucose bouillon tends in the end to favor *B. acidophilus* against enterococci.

CONCLUSIONS

1. Enterococci were found to exist in the digestive tract of normal calves. They were also present in varying numbers in calves suffering from diarrhea, or scours. They sometimes developed in practically pure culture at certain levels.

2. The enterococcus organisms from calves do not represent a homogeneous group but are separated into four divisions according to fermentation reactions. These groups agree with the type form and certain variants described by Dible and by Kendall and Haner.

3. All the calf strains examined agreed in coagulating milk, leaving gelatin intact, producing green zones on blood agar plates, fermenting glucose, lactose, maltose, and salicin, and maintaining the same range of H-ion concentration for optimum growth. They differed in the fermentation of mannitol, sucrose, raffinose, and inulin; also in capsule formation, in the character of sediment in bouillon, in the degree of resistance to heat, and in the limiting H-ion concentration.

4. Enterococci growing in mixed cultures with *B. coli* are able to survive *B. coli* whenever acid conditions are produced. Enterococci are also harmful to *B. acidophilus* when they are grown together in mixed cultures, unless the early development of the enterococcus is delayed. In this case the injurious effect is not due to acid but rather to the rapid multiplication of the enterococci. Whenever acidophilus bacilli survive in such a mixed

culture they are found later in the tubes containing acid; thus the acidity tends in the end to favor *B. acidophilus* against enterococci.

5. The enterococcus differs from *B. acidophilus* in its ability to grow in a more alkaline medium, and also in being slightly less resistant to acid, although it is more resistant than *B. coli*.

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THE USE OF POTASSIUM TELLURITE IN DIFFERENTIAL MEDIA[1]

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The chemical and pharmacological properties of tellurium have long been a matter of interest to investigators. Early work groups tellurium and the closely related substance, selenium, with arsenic and antimony in their effect on the mammalian organism. These substances belong to the intermediate group of elements, which are capable of functioning as bases or as non-metals. Tellurium, like selenium, when it is a base, is usually bivalent (H_2Te), but in tellurious acid it is tetravalent (H_2TeO_3), and in telluric acid, hexavalent (H_2TeO_4). A resemblance can be seen to the similar compounds of sulphur, to which tellurium and selenium are closely related chemically and physically.

Gies, and Mead and Gies, in 1901, reviewed earlier work and discussed the physiological and toxicological effects of tellurium in detail. They found that tellurites were readily reduced to metallic tellurium by living protoplasm, and that granular deposits of the metal were widely found in the tissues of animals killed by the administration of toxic doses of tellurite.

Knop (1885) and Bokorny (1893) found that sprouting maize, algae and infusoria are uninjured by concentrations of potassium tellurite as low as 0.1 per cent. Jones (1909), in a study of selenite, found that heated tissue does not lose its ability to reduce the salt, and concluded that the active agent was a reducing sugar. He demonstrated *in vitro,* that glucose and related reducing substances readily caused the precipitation of metallic selenium at 35°C. and higher temperatures.

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Scheurlen (1900) and Klett (1900) extended the study to bacteria. Scheurlen found that *B. anthracis* and other bacteria were colored by reduced metal when cultivated in the presence of salts of tellurious and selenious acids. Klett studied the growth of numerous bacteria and of molds under the influence of compounds of selenium and tellurium. He found that the development of molds and of certain bacteria was not materially affected by the presence of small quantities of sodium tellurite, while that of certain other organisms was strongly inhibited. Some types seemed more susceptible to tellurites, some to selenites. This author also noted the reduction phenomenon, and stated that the formation of intracellular granules was proportional to growth. He found that selenate was relatively inactive and concluded by analogy that tellurates would be less active than tellurites.

Klett (1900) mentions *B. diphtheriae* as one of the organisms employed by him in his study. In the quantities that he used (not measured accurately), growth was slower than in controls, but after several days a reduction of the salt was observed. However, many other organisms were much more strongly inhibited.

Conradi and Troch, in 1912, attempted to use these salts for differential purposes. A modification of Loeffler's serum medium containing a small quantity of calcium malate and potassium tellurite (Te_4 concentration $\pm$ 1:10,000) was used. This medium is stated to be inhibitive for many saprophytes, and to hold overgrowth effectively in check. In addition, the colonies of *B. diphtheriae* and related organisms are easily selected, since they are strongly reductive. These authors found the blackening, although not specific, to be a characteristic feature at the close of the usual incubation period (eighteen to twenty-four hours), when colonies of other types usually failed to show reduction.

Smith, in 1914, compared the differential antiseptic action of potassium tellurate (Te_6 $\pm$ 1:18,800), and tellurite. He found the action of the latter to be less marked. This conclusion does not seem to have been confirmed by others, the weight of evidence being in favor of the greater activity of tellurites. The phenomenon of reduction was not emphasized by this medium, as

the author reported that the colonies of *B. diphtheriae* were white. The basic medium is an agar containing 5 per cent of serum. Smith states that this quantity is the optimum, since the use of a smaller amount gave less vigorous growth, while more decreased the differential antiseptic effect of the salt.

In 1921, Joachimoglu and Hirose determined the concentrations of the tellurates, tellurites, selenates and selenites of sodium tolerated by *B. diphtheriae*. An emulsion of organisms grown on Loeffler's medium was inoculated into tubes of glucose broth containing varying dilutions of these salts, and after twenty-four hours at 18 to 22°C., transfers were made to a suitable medium. The following concentrations were destructive under these conditions: Te_4 (as tellurite) 1:420; Te_6 (as tellurate) 1:125; Se_4 (as selenite) 1:1160; Se_6 (as selenate) 1:666. The comparatively high concentrations of these salts, tolerated by *B. diphtheriae* are in marked contrast to those effective for the destruction of many of the organisms tested, and this fact gives an explanation of the differential value of compounds of selenium and tellurium. An illustration of this wide difference is seen in the comparison of the effect of tellurite on *B. diptheriae* and *B. typhosus*. The former tolerates a concentration of tellurium about 4000 times greater than the latter. On examination of the figures given above, it will be noted that, contrary to the experience of these authors with most of the organisms tested by them, selenium is more toxic than tellurium for *B. diphtheriae*.

Douglas (1922) reports excellent results in the isolation of *B. diphtheriae* on a plating medium consisting of a trypsinized serum agar containing tellurite (Te_4 1:6000). He employs 10 to 15 per cent of a serum of which the antitryptic activity has been previously neutralized by the addition of trypsin. On plates examined at the end of eighteen to twenty hours, *B. diphtheriae* and related organisms show a very characteristic darkening and a granular appearance. Few other organisms found in throat cultures give a similar picture at this stage.

Megrail (1923) has found a serum agar containing 1:20,000 potassium tellurite very satisfactory for the isolation of diphtheria bacilli.

Pergola (1921; 1922; 1923) is reported as using several combinations of potassium tellurite with agar, gelatin, serum, etc. He also employs, as accessory to his plating media, an enrichment serum broth containing tellurite. Since the original articles are not available it is impossible to examine his methods in detail. However, the conclusions state that tellurite is effective as a selective, inhibitory agent, and that the use of tellurite media permits the isolation of *B. diphtheriae*, even when present in small numbers. The author states that selenium cannot be used as a substitute.

Greenspon (1923) and Hill (1922) have recently advocated the use of a moderately acid reaction (pH 6.6$\pm$) as favorable for *B. diphtheriae* and inhibitive for other organisms.

This review of the literature indicates the marked value of certain tellurium compounds in differential media, especially when used for the isolation of diphtheria bacilli. As media containing tellurites have not been extensively used in routine laboratory work in this country, it has been thought that a report of our experience with a medium similar to those recommended by Douglas (1922) and Smith (1914) might be of interest.

The routine procedure that had been employed in this laboratory to isolate *B. diphtheriae* for the purpose of determining virulence was as follows: A representative portion of the original growth on Loeffler's medium was emulsified in 20 per cent ascitic-fluid broth, and the emulsion plated in series on two plates of medium containing 2.5 per cent agar, 0.2 per cent glucose, and 5 per cent horse serum. The plates and the slanted tube were incubated. The tube of broth served as an enrichment medium, its value resting on the fact that if diphtheria bacilli are present a pellicle may be formed. After eighteen to twenty hours, another plate culture was made from this enrichment broth, a fragment of the pellicle being used, if present, or if not, a loopful of broth, taken from the surface region. This plate was fished on the succeeding day if the first plates had not permitted the isolation of *B. diphtheriae*. The colonies on this plating medium were small, but were much more readily fished than from a similar medium without serum.

Since the plating medium used in this laboratory approximates the basic media of Smith (1914) and Douglas (1922), it was used as the starting point for this study.

Tests were made to determine the optimum amount of tellurite—that which would permit the maximum inhibition of other organisms without decreasing the size of the *B. diphtheriae* colonies—and to determine whether or not tellurite, in the concentration selected, affects the virulence of the strains isolated.

A few tests were made to gain preliminary data, the results of which may be summarized as follows:

1. Tellurium in concentrations of 1:34,000 and 1:36,000 permits satisfactory growth of *B. diphtheriae* on an infusion glucose-serum agar base, while 1:20,000 and 1:22,000 concentrations are somewhat inhibitory.

2. Colonies of *B. diphtheriae* on media containing 1.5 per cent agar are more characteristic than those on the routine 2.5 per cent agar plating medium. The heavier agar had been used in the routine since it tended to inhibit the rapid spread of motile, contaminating organisms.

3. Colonies of *B. diphtheriae* are fairly distinctive on this medium, being small, granular and "smoky" after eighteen to twenty-four hours' incubation.

A supply of 1.5 per cent agar with 5 per cent horse serum and 0.2 per cent glucose was prepared with approximately 1:8000, 1:17,000, 1:34,000, 1:50,000, 1:66,000 and 1:100,000 parts of tellurium.

No growth of *B. pyocyaneus* or *B. subtilis* was obtained on any of these media after twenty-four hours' incubation. Three cultures of virulent diphtheria bacilli and a culture of *Staphylococcus aureus* grew well on all of the plates. The colonies of the diphtheria bacilli were smaller on the medium containing 1:8000 tellurium and in some cases on that containing 1:17,000. On all of the other plates the colonies were of approximately the same size. More blackening occurred, however, on the medium containing lower dilutions of tellurite.

It was found that the medium, when prepared as described, frequently became contaminated with molds. As previous work

had shown that a tellurite solution cannot be sterilized by heat, it was decided to sterilize the solution by filtration. A supply of potassium tellurite solution, 1 per cent by weight, approximately 0.58 per cent by actual test, was therefore prepared. This solution was passed through a Mandler filter. Chemical tests showed the solution tube very slightly concentrated after the filtration, in one determination it being 0.577 per cent before passing through the filter and 0.583 per cent after filtration. Media prepared with the filtered solution proved satisfactory.

After these preliminary tests it was thought advisable to compare the efficiency of the medium containing tellurite with that previously employed in the routine for the isolation of

TABLE 1

*Results obtained with both routine and tellurite media from March 18 to August 18**

	B. DIPHTHERIAE	B. DIPHTHERIAE LIKE	TOTAL
Isolated with tellurite plates only	22	6	28
Isolated with routine plates only	12	9	21
Isolated with both	43	16	59
Unsuccessful with both	0	0	25
Total	77	31	133

* In case atypical organisms are found in cultures submitted for routine examination, an attempt is made to identify the diphtheria-like bacilli. From many of such cultures nothing of significance is isolated.

diphtheria bacilli from throat cultures. The two media differed only in that the tellurite medium contained 1.5 per cent agar and approximately 1:17,000 parts of potassium tellurite, while the routine medium contained 2.5 per cent agar. The results of examinations during a period of five months are shown in table 1.

In 24 of the 43 cases in which diphtheria bacilli were isolated from both routine and experimental media, the percentage of successful fishings was higher with the experimental media; in 10 cases the percentages were equal.

So far as can be determined, the presence of potassium tellurite in the medium has no appreciable effect on the virulence of the diphtheria bacilli isolated. Of the 22 strains isolated on the tellurite medium 17 proved to be virulent and 5 proved to

be non-virulent. Thirty-four cultures that had been isolated on the routine medium and found to be virulent were replated on a medium containing approximately 1:34,000 parts of tellurium. Colonies were fished after twenty-four hours and the virulence test was repeated. All of these cultures were still virulent.

Colonies of *B. diphtheriae* on the tellurite medium are not difficult to distinguish and can be fished microscopically with success when they are present in moderate numbers. They are white, or white about the periphery with grey centers, the depth of color increasing with the period of incubation up to forty to forty-eight hours. Staphylococci are entirely black, or black in the center with a narrower light periphery. Microscopically, the diphtheria colonies are round, coarsely granular, with dull, dark or "smoky" raised centers. The periphery is flat and lighter with unbroken or very slightly irregular edges. Microscopically, colonies of staphylococci are of about the same size or somewhat larger than colonies of *B. diphtheriae*, but are black, highly refractive, round, smooth and raised, with edges entire. Colonies of *B. hoffmanni* resemble those of *B. diphtheriae* but have a heavier periphery. Streptococcus colonies are not always characteristic. Microscopically, they may be confused with those of *B. diphtheriae* as they are white, or white with greyish centers. They are smaller, however, and microscopically are flat with a slight brownish coloration of the center.

There are some other factors which should be considered in a study of this sort, such as the keeping quality of the medium and of the stock tellurite solution. Smith (1914) states that tellurate gradually loses its antiseptic action. The ease with which reduction occurs suggests that the tellurite solution is relatively unstable. We have found, however, that a medium containing tellurite solution that had been filtered through a Mandler filter twenty days before use proved entirely satisfactory. Medium in plates that has been left eight days at a temperature of 3° to 6°C. has proved somewhat less satisfactory than that freshly prepared, the colonies of diphtheria bacilli being somewhat smaller on the former medium. Growth of *B. pyocyaneus* and *B. subtilis* was completely inhibited on it however. The smaller

size of the colonies of diphtheria bacilli may have been due to the fact that the surface of the medium that had been stored was dryer than in the case of that which was freshly prepared.

TABLE 2

Growth of different organisms on medium containing tellurite 1:34,000

LITTLE OR NO INHIBITION	SLIGHT INHIBITION	MARKED INHIBITION	NO GROWTH
Streptococcus hemolyticus	Pneumococcus Type I	*Micrococcus meningitidis*	*Micrococcus catarrhalis*
Staphylococcus aureus	Pneumococcus Type II	*Sarcina lutea*	*Micrococcus gonorrheae*
Micrococcus tetragenus	Pneumococcus Type III	*Bacillus dysenteriae*, Shiga	*Bacillus abortus*
Bacillus acidophilus	*Bacillus cholera suis*	*Bacillus dysenteriae*, Flexner	*Bacillus anthracis*
Bacillus coli	*Bacillus morgani*	*Bacillus dysenteriae*, Mt. Desert	*Bacillus anthracis symptomaticus*
Bacillus diphtheriae	*Bacillus mucosus capsulatus*	*Bacillus enteriditis*	*Bacillus botulinus* A
Bacillus hoagii	*Bacillus pertussis*	*Bacillus lactis aerogenes*	*Bacillus botulinus B*
Bacillus hoffmanni	*Bacillus rhinoscleroma*	*Bacillus paratyphosus* A	*Bacillus fecalis alkaligenes*
Bacillus influenzae		*Bacillus paratyphosus* B	*Bacillus fluorescens*
Bacillus proteus vulgaris		*Bacillus pestis caviae*	*Bacillus histolyticus*
Bacillus proteus X19		*Bacillus suipestifer*	*Bacillus mallei*
Bacillus welchii		*Bacillus typhosus*	*Bacillus melitensis*
Bacillus xerosis			*Bacillus oedematis maligni*
Spirillum cholera			*Bacillus prodigiosus*
Actinomyces bovis			*Bacillus pullorum*
Aspergillus niger			*Bacillus putrificus*
Saccharomyces cerevisiae			*Bacillus pyocyaneus*
			Bacillus sporogenes
			Bacillus subtilis
			Bacillus tetani
			Bacillus typhii murium
			Bacillus violaceus

After making the tests with diphtheria bacilli, it was thought well to learn with what other types of organisms the medium

would be useful. A number of cultures from the stock collection were tested on a medium containing approximately 1:34,000 tellurium. Table 2 shows the character of the growth obtained (for *B. influenzae* and *B. pertussis* blood extract agar was employed). These results indicate that media containing potassium tellurite may be valuable in the isolation of Gram-positive cocci, certain of the higher bacteria, molds and yeasts, and possibly in the isolation of *B. welchii* from mixtures with some of the other anaerobes.

The method of preparing the medium is as follows:

Beef infusion agar 1.5 per cent..........	1 liter
Horse serum (sterile)....................	5 per cent or 50 cc. per liter
Glucose C. P., 20 per cent solution (sterile)..................................	1 per cent or 10 cc. per liter
Potassium tellurite C. P., 1 per cent solution (0.58 per cent on test)............	1 per cent or 10 cc. per liter 1:34,000 Te_4 (1–17,000 K_2TeO_3)

Procedure. Melt the agar and cool to 50°C. Add the serum glucose and tellurite solutions, all previously warmed to 50°C. Mix well and insert a sterile siphon. Dispense in Petri dishes. Incubate the plates and examine for contaminations before use.

The solution of potassium tellurite is prepared as follows:

Potassium tellurite C. P..................................	0.25 gram
Distilled water..	25.0 cc.

Procedure. Weight 250 mgm. K_2TeO_3 on a chemical balance. Grind to a very fine powder in a small mortar and add about 10 cc. of the water. Mix well, allow the undissolved portion to settle and pour off the supernatant fluid. Add more of the water to the residue, grind again and combine the two portions. Rinse the mortar with the remainder of the water.

The solution is then passed through a paper filter and then through the Mandler to sterilize it.

Note. It is difficult to get this compound into solution and even after thorough grinding in the mortar, a small amount of fine suspension is visible. Heating gently to about 55°C., aids the action somewhat.

As was indicated in the preliminary tests the range of the tellurium content can be wide. It has been thought advisable,

however, to determine the approximate amount of the salt that will dissolve, especially when a new supply is purchased. The method described by Gooch (1912) and Gooch and Donner (1892) has proved satisfactory.

SUMMARY AND CONCLUSIONS

A concentration of potassium tellurite approximating 1:17,000 gave excellent results in the isolation of diphtheria bacilli from throat cultures, and seemed to have no effect upon the virulence of the organisms. From the observation of the growth of a number of cultures in the stock collection, it was found that certain Gram-positive cocci, some of the higher bacteria, yeasts, and molds seemed to be inhibited only slightly if at all, while many of the motile bacilli, including a number of anaerobic organisms, were markedly inhibited by potassium tellurite in the concentration mentioned.

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OUR SOCIETY—IN RETROSPECT AND PROSPECT[1]

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From all the facts gathered concerning prehistoric man, the inferences show that he was a social animal. By force of many circumstances, physical and environmental, he was bound to be so in large degree, lest the forces of nature and his environment should have overwhelmed one so apparently unfitted to survive. It was in great measure the cultivation of his social instincts, I am convinced, that outweighed the adverse balance in nature against him and preserved the race.

With the passage of the thousands of years, this instinct has been preserved and accentuated. From its service as a purely protective measure, it has developed in a variety of ways, conserving customs, regulating habits, modes of living and of thought, and stimulating the growth, intercourse and exchange of ideas. In this latter quality of the primitive impulse as developed today, we find the cause of the grouping of civilized man in social circles under the guise of secret societies, benevolent societies, religious beliefs and scientific associations. In this latter group the old primitive force has found its widest expression, as witness the facts, that its bounds are neither sectarian nor national, but international, world-wide, giving the largest opportunity to mankind to reach its highest goal in aspirations, the full comprehension of truth and knowledge. In forwarding such aspirations, our Society is contributing its share.

To attempt a full résumé of our activities, past and present, is not the object of my address, but it might be well in this regard to make a few remarks for the benefit of our recent members, and

[1] Presidential address delivered at the twenty-seventh annual meeting of the Society of American Bacteriologists, Madison, Wisconsin, December 30, 1925.

to polish up the memories of the older ones, to show when and how the spell of the primitive urge manifested itself in our relations.

The Society of American Bacteriologists was founded in 1899, and held its first meeting at Yale University during the Christmas vacation of that year by the united action of some members still living and of others who have passed along. At the time it was felt that there should be some adequate channel of expression and interchange of ideas in the field of bacteriology, which had now in this country arrived at the definite status of a science, with the aim in mind to push forward its boundaries both in pure science and in the field of art and practice. The enthusiasm of those early days, the subjects discussed, debates on the papers and the social communion enjoyed, some of us can still recall. The uplift was felt by all, and pride in our organization was mighty. The years have passed and we still flourish, and our pride and enthusiasm, I take it, are not one whit diminished. From these small, but energetic beginnings of those early years our Society under the able guidance of such Presidents, as Sedgwick, Welch, Conn, Th. Smith, Novy, Jordan, Erwin Smith, Carroll, H. L. Russell, Kinyoun, Moore, Gorham, and of untiring, self-sacrificing secretaries as H. W. Conn, Marshall and Hitchens, our Society became solidly established, and has continued onward in the path of progress—a Society for which we today entertain feelings of the warmest regard and pride.

Touching now upon some of our outstanding achievements, your attention is directed to the following remarks:

One of the earliest ventures on our part, was the publication of the Society's "Descriptive Chart for the Identification of Bacterial Species," founded originally on previous efforts by the late Professor H. W. Conn and Dr. S. de M. Gage. This work was largely due to the efforts of Dr. F. D. Chester in 1903 to 1904, and a Committee on Methods for the Identification of Bacterial Species was formed with Dr. Chester as Chairman, his place being eventually taken by Professor Erwin F. Smith in 1907 when the chart was perfected and sponsored by the Society. In following years under various Chairmen, the chart underwent constant revision and has proved of wonderful assistance to all workers.

Out of the activities of this Committee, which was dissolved later on, grew the Committee on the Descriptive Chart, and in turn in 1920 a new Committee called the Committee on Bacteriological Technic was formed which had a broader field of activities accorded it and was placed under the able guidance of Dr. H. J. Conn who yet remains at the helm. This Committee has been responsible for the appearance of the admirable "Manual of Methods for Pure Culture Study of Bacteria" of which the Society is so justly proud.

Then in 1916 came the founding of the "Journal of Bacteriology" and in 1917 "Abstracts of Bacteriology," under the management of the Editors, Dr. C.-E. A. Winslow and Dr. A. P. Hitchens, respectively, and of an editorial board. Through the unceasing labors and skill of Drs. Winslow and Hitchens these publications have brought the Society international recognition.

Feeling the necessity of a more modern treatise on bacterial species than that useful book published by Dr. F. D. Chester in 1901, a Committee on Characterization and Classification of Bacteria was formed in 1917 under the Chairmanship of Professor D. H. Bergey, whose painstaking and highly commendatory efforts were published in book form in 1923, under the title of "Bergey's Manual of Determinative Bacteriology." In this field also the Committee on Taxonomy functioned under the excellent Chairmanship of Dr. C.-E. A. Winslow, and has published several papers by its members, in particular by Professor R. E. Buchanan, who under the aegis of the Society, has published this year the first volume of "Monographs on Systematic Bacteriology," which has received a very warm welcome throughout the English-speaking scientific world—and in turn, the reputation of the Society has been equally lustered.

In 1922 steps were taken to transfer the Type Culture Collection of Bacteria from the American Museum of Natural History, where it had been established by Dr. Winslow a few years previously, and to make it a part of our activities. This was accomplished and the Collection housed at the Army Medical Museum under the care of several of our indefatigable members. On account of unforeseen administrative difficulties it appeared

possible that the Society would have to abandon this one of its ventures, but a year ago, thanks to the generosity of the Director of the John McCormick Memorial Institute, of Chicago, Dr. Ludvig Hektoen, the Collection has found a permanent home under the efficient curatorship of Dr. Geo. H. Weaver.

Through one of our members, Dr. H. J. Conn, acting as Chairman, the Commission on Standardization of Biological Stains, sponsored by the National Research Council, has this year given us a splendid reference-book under the title of "Biological Stains," which will go far towards directing the attention of the new dye industry in this country to the needs of the biological sciences, and at the same time presenting all laboratory workers with a critical and comprehensive compendium which will give them a more intelligible knowledge of certain of the tools they work with. And, of course, you are already well aware of the very practical work this Commission is doing in certificating the validity and purity of biological stains made by American manufacturers.

But with the passing of time, it strikes me, in spite of our past accomplishments, that our marching step is not quite tuned up synchronously to the tramp of the legions of science in other spheres of knowledge. In this view I may be pessimistic, but with the passing of one's youthful enthusiasms, one is so prone to view present-day affairs in such a light, and I trust that you will pardon my croaking and bear with me.

Apropos of my remarks a moment ago, dealing with the early formative years of the Society, we must recognize that although we are only twenty-six years old, older perhaps than some of the members sitting before me tonight, we already possess a historic background and should take steps to preserve and add to it. So looking forward to the time when many of those intimately concerned in the initiative and struggling days of the Society will be passing off the stage, we should be up and doing, lest data easily available now be permanently lost to our records. Already, I am given to understand, documents of historic value are at this moment in other hands than those of the Secretary, where naturally they ought to be. It is believed that they are yet accessible, and every effort should be made to secure them.

All such material should without delay be collected, sifted and documented.

For an already overworked officer, this would prove to be impossible were he ever so willing, so to this end I offer the suggestion that there be permanently appointed, through proper channels, a member to take on the duties of Archivist or Historian, who, together with the Executive Officers and Council, would collect, evaluate and file all documents of possible historic importance, discarding all material reckoned to be worthless. This procedure might be repeated say every fifth year, in reference to further accumulations, and so there could be ensured a continuity of action throughout our existence, and give to further generations of members a glimpse of our progress from small beginnings. I can also conceive that our archivist might prove himself to be very human and entertaining if he were called upon to add to the zest of the Annual Dinner by giving, once in a while, brief discourses upon topics exhumed by him from the musty files under his care!

Our clothes of earlier years, having become too small for us, our Constitution underwent a much-needed revision in 1921, and again we are listening to complaints that we need a new suit. If this be so, by all means call in the tailor and trust that the subject of rehabilitation will be thorough and not long delayed. In this connection I would advocate the appointment by the President, of a standing Committee on Revision of the Constitution to whom members could forward suggestions which would then be dealt with on their merits and action taken through the usual channels.

In any contemplated revision of the by-laws, I would advocate the appointment of the Chairman of the Program Committee to be for a period of three years. Such a change would lead to a definite continuity of method in the framing of the annual program and would, I am confident, lead to the establishment of a permanently better balanced plan for annual meetings than at present prevails. Elsewhere, I have urged reform in methods of program planning, and perhaps a word or two might not be amiss here in this connection. I am convinced that provision should

be made in the program covering both the general meetings and each of the section meetings to enable the members to listen to one paper prepared, by request, by someone an authority on some topic of particular interest, followed by discussion prepared in advance by one or two members, then general discussion by anyone in the audience. Such a plan would lead to a thorough ventilation of the selected topic, and members in general would carry away with them a store of accepted opinion of the greatest value. Present day program planning such as we have pursued, makes no provision for a carefully prepared presentation of a subject of general or special interest.

Before leaving this subject of the program, another thought presents itself that might be given consideration, even though it may seem to tread upon the sensitive toes of custom. In many of our scientific societies at the present day, the opening number of the program is devoted to the President's address. This appeals to me very strongly as being the logical and appropriate time and place for it, rather than at the dinner where distractions of one sort or another tend to interfere with its presentation, and where at times the acoustic properties of the place sadly mar the pleasure of the listeners. To take the place of the presidential address at the dinner, I would suggest the organization of two or three brief orations by certain gifted members of the Society, or from one or two especially invited guests; such a departure, I anticipate, would in no wise detract from the pleasures of members accustomed to the old order.

With the steady growth of the Society in later years, its several activities have likewise expanded, so that it is plain to most of us that the time allotted to the business section of our program renders it now almost impossible to properly give the requisite degree of careful discussion to the multiplicity of business affairs, which constitutes so vital a part of the Society's fabric. To amend this state of affairs which is becoming each year more serious, I would advocate allotting one morning session to the business meeting, or even going the length of sacrificing a portion of that part of our program now given over to social functions. That such a move is radical, I do not deny, but the sub-

ject is one that is not to be denied and will sooner or later have to be faced by future executive officers.

In certain communities of men, as the result once more of the inherent primal social instinct, tradition often plays an important rôle in their development and progress. In some cases it proves highly beneficial to the community involved; in other instances it leads to cessation or retardation of progress; particularly is this so where tradition becomes a fetish, a thing to be worshipped, reverenced and hedged about. Tradition rightly evaluated is an object to be cherished, and though certain modes in our Society have been recognized and adhered to during the years of our existence, I scarcely think that we have yet earned the right to regard them in the light of anything traditional. So I venture again to offer a suggestion, which I feel, if tried out, would lead to giving added zest and interest to our annual gatherings. This would be the organization of what is known in some quarters as the "Round Table Conference." It is an informal gathering in the widest sense, and from personal knowledge of its operation I have formed the opinion that through its broadness of character, its freedom of expression, the wide range of topics touched upon in discussion, it would constitute a most valuable addition to our activities, particularly interesting to everyone, whether he listens in or actively participates. The meeting would be presided over by a chairman previously appointed in the general business meeting, who would with as little formality as possible, conduct proceedings. To cover as much ground as possible, ten minutes should be allotted to the speaker introducing his subject, and five minutes to those carrying on discussion. This "Round Table Conference" could readily enough take up one hour of the time usually set aside for the "smoker," thereby adding considerable profit to the entertainment.

I touch now upon another side of our activities, one which has caused all members considerable anxiety in the past and still exists to give us concern, namely, the question of keeping up our membership to such a level that cause for financial worry will cease. Some few years ago, under the very active chairmanship

of Mr. S. H. Ayers, an intensive campaign for membership was undertaken which was fruitful of immediate results, but was almost as soon counterbalanced by resignations in other instances, and today our numbers show only a slight advance. It is a discouraging situation, and one for which there does not appear to be an adequate solution at the present moment. I simply mention it here in the hope that each member will make up his or her mind by personal effort in any likely quarter to secure suitable additions to our membership. In connection with this question of membership, it seems to be an opportune moment to offer a suggestion which might go far to lessen the annual number of resignations. Let us consider that owing to our yearly change of meeting-places, we can at best count only upon an attendance of about 33 per cent of our total membership at any one assembly; of this number the percentage of regular attendants is by no means small. This then leaves a relatively high percentage of members who do not attend a meeting, say, once in four or five years. Amongst these loss of interest in the affairs of the Society insidiously creeps in, and this attitude of mind passes on into cold indifference, ultimately leading up to resignation. To keep up a vital interest amongst absentees, from whatever cause, I feel that the Society should make an effort to distribute among them a short report of its annual meeting. All that is necessary would be to publish as soon after the meeting as possible, a brief synopsis of the business session, together with any other items of interest apart therefrom. Let the bulletin be in any form so long as its contents can make such an appeal to the absent member that he feels that he is not losing touch with the Society and its affairs. The cost of printing and distribution cannot be very great, and the results accruing therefrom would, I am confident, justify the expense.

Directing our thoughts now into another channel, I cannot refrain from touching upon one of the phases of the late war, in as much as it holds forth a lesson for us. None of us who joined up, either as a "buck" private, an officer, or of whatever rank, failed to quickly perceive the great necessity and value of organization. Those of us who rendered special services on account of

our previous technical training were probably more impressed by this necessity for organization, sometimes of a most delicately balanced coöperation, where close liaison with persons in other services was demanded. I need not quote individual instances where undoubtedly the winning of the war in the last analysis, depended upon the results attained by such careful coöperation.

That such knowledge has since become widely diffused in civil life by those whose fortune it was to be called to the colors, is well known, and, what is more to the point, there is considerable evidence before us that such lessons gained in the armies have not passed out of mind unheeded, but have been and are being either advocated or applied in business, professional and scientific circles today. So I would now direct your attention to some examples and quotations dealing with the value of coöperative work in research, quite apart from any military reference and leading up to what I wish to advocate as adding possibly another to the several activities carried on by us.

To begin with, the brilliant discovery of insulin in 1921 to 1922 by Banting, Best and Macleod at the University of Toronto, constitutes an outstanding early example of success in research through the associated efforts of chemists, physiologists and clinicians. Its immediate success measured in the number of lives saved, and its tremendous stimulus given to group-research can hardly be imagined.

In the attempt to unravel the mysteries surrounding the activities of the human strain of the tubercle bacillus, a further splendid example of group organization in research is exemplified by the Research Committee of the National Tuberculosis Association under the direction of the Chairman, Dr. W. C. White of the United States Public Health Service at Washington. He has succeeded in enlisting workers in several of the seats of learning in this country to attack phases of the problems by specialists in bacteriology, chemistry, histology, pathology, clinical medicine and pharmacology. To my knowledge this constitutes the greatest modern group-effort in scientific research yet undertaken, and truly points the way for others to follow in.

Dr. Florence Sabin, now one of the Research staff of the

Rockefeller Institute, formerly a leading authority on research problems in the field of anatomy at the Johns Hopkins Medical School, in the course of her presidential address given before the American Association of Anatomists this year, speaking of some aspects of research, bearing upon my remarks, says:

"If you compare the titles of scientific articles in the journals of today with those of twenty-years ago, you will find that we are beginning to work in groups, often quite large groups. The nature of research is changing in such a way as to make this necessary." Continuing she cites the example, just mentioned, of the Research Committee of the National Tuberculosis Association, and the coöperation of chemists, histologists and clinicians in the recent and successful attack upon rickets.

A notable contribution to this theme has recently appeared in "Science" by Professor Chauncey D. Leake of this University, within whose walls we are meeting, wherein he demonstrates as an outcome of the necessities of war-time measures, the value of group research. The lessons so learned have resulted in the now well-known series of excellent studies upon the value of tryparsamide in the treatment of neuro-syphilis by himself, Professor Loevenhart and associates.

In stressing this modern movement, I do not for one moment want to be understood as advocating pushing into the background, the development and activities of the individual research worker. For from it, because it is so self-evident that the very successes to be gained through group-research depend so vitally upon combinations of persons of proven high attainment in research. But since science has become so increasingly complex, one may readily envision the fact that it is now beyond the powers of one individual to encompass some problems whose ultimate solution may lie scattered amongst a number of branches of science.

Without further elaboration of this subject, I beg to offer a proposal to the Society that as one of its future ventures in forwarding our science, it undertake at some early date the formation of a committee, which might be known as the "Research Conference Committee," whose duties it would be, first, to formulate some topics considered by it as worthy of intensive group-

effort; second, to stand ready and willing to receive and consider for action suggestions of the same sort submitted by any member; third, to invite certain well-qualified members to participate in the research; fourth, through executive channels to solicit financial aid, where necessary, from quarters where grants are already made available, or from new sources (see Bull. Nat. Res. Council, 1921, II, part I, No. 9); and fifth, to publish results of such research, as work done under the authorization of the Society. Such an endeavor in this newly developing method of investigation would tend to elevate our Society still further in the estimation of its fellows in science, and detract in no whit whatever from the reputation of those of its members, or others outside of the Society associated with them, in any coöperative venture into the realms of the present vasty unknown. Some may feel that research is a matter for individual effort alone, then to them my argument can carry no appeal, but surely, as I intimated before, there are extant many problems in bacteriology that today no one worker can ever hope to solve, but which await coöperative study. One comes to mind in regard to the phenomenon of bacteriophagy, which despite d'Herelle's brilliant researches and writings leaves many unconvinced as to the real cause and operation of so interesting a phenomenon. Under present conditions of individual research, the study of this phenomenon has led to discordant opinions regarding it, and years of scattered effort and argument may pass before an acceptable solution of it be established; studies in mutation offer another field for group effort; the etiology of influenza another, and so on.

Such as they are, these reflections of mine concerning the state of our "body politic" may, I hope, be acceptable of consideration. They are given in the conviction that our Society cannot remain in a static state, be that state ever so excellent. If we are thoroughly vitalized, then we must press forward towards newer accomplishments, and it is with such a point in view that my address, however unworthy, has been formulated; paraphrasing that so well-known phrase of Horace, I may say that "*tempora mutantur et nos mutandi summus in illis.*"

I cannot bring my words to a conclusion without expressing

to you all the warm feelings I entertain towards the members of the Society for having in the past year so highly honored me with their confidence in making me their chief executive officer; and in laying down the trappings of office, I beg to extend to all the other executive officers and Council my hearty thanks for their cordial coöperation with me, and particularly to that weary Atlas, our indefatigible Secretary, whose shoulders carry no light weight of constant responsibility—ours the glory, his the toil.

The annual report of the committee in charge of the American Type Culture Collection, shows that the available cultures have increased from 175 to 722 during the period February 1 to December 15,1925, including 69moulds and 122 yeasts. A total of 360 orders has been filled, involving the sending of 1540 cultures to 240 persons and institutions. The committee has established a price of one dollar per culture with addition for packing and postage. A temporary list of cultures is available. A printed catalogue is planned for the near future. Communications should be sent to The American Type Culture Collections, John McCormick Institute for Infectious Diseases, 637 S. Wood Street, Chicago, Illinois.

THE MAGNITUDE OF THE ERROR DUE TO AMMONIA AND ITS SALTS IN THE VAN SLYKE AMINO NITROGEN PROCEDURE AS COMMONLY APPLIED IN STUDIES OF BACTERIAL METABOLISM

L. B. PARSONS AND W. S. STURGES

From the Laboratory of the Cudahy Packing Company, Omaha

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Although Van Slyke definitely states that the presence of ammonia may cause a serious error in the determination of amino nitrogen by his method, many bacteriologists have apparently failed to appreciate the importance of this point. He warns that,

> For the accurate determination of amino nitrogen in digesting solutions, etc., it is advisable first to remove ammonia although good comparative results can be obtained in the presence of the relatively small proportion of ammonia usually present, if the reaction conditions of time, concentration, and temperature of solutions are kept constant so that the proportion of the ammonia decomposed is the same in each determination.

He even presents some data (table 1) showing the amounts of nitrogen liberated from an ammonium sulfate solution when allowed to react for various lengths of time.

A brief survey of recent studies of nitrogen metabolism of bacteria (Wagner Dozier and Meyer, Avery and Cullen) failed to show that these investigators had made any allowance for ammonia interference in their Van Slyke amino nitrogen determinations. Others (De Bord and Lamson) have criticized and discarded the method without considering the importance of the ammonia interference.

Kendall recognized the indefiniteness of the ammonia correction and therefore gave preference to the Sörensen method

for amino nitrogen. Reddish and Rettger apparently recognized the ammonia error since they removed the ammonia before analyzing for amino nitrogen by the Van Slyke method.

In view of the conflicting experiences of various investigators, it seemed worth while to undertake a brief physico-chemical investigation of the nature of the reaction of ammonium salts in the Van Slyke procedure, and to determine the magnitude of the error under various conditions.

TABLE 1

Used 10 cc. portions of ammonium sulfate solutions containing 28.02 mgm. of nitrogen. Temperature 24. Pressure 752 mm.

TIME OF REACTION	NITROGEN	WEIGHT OF NITROGEN	PER CENT OF TOTAL AMMONIA NITROGEN
minutes	*cc.*	*mgm.*	
3	12.1	6.86	24.5
5	18.4	10.16	36.3
10	31.5	17.38	62.1

EXPERIMENTAL

The experiments detailed in this paper were carried out with the micro-apparatus. Reagents of a satisfactory degree of purity were used. The sodium nitrite solution used was made up to the recommended concentration of 300 grams per liter, unless otherwise specified. The ammonium salt solutions used were made from the purest materials available and the concentrations were verified by subsequent analysis for ammonia.

The quantities of acetic acid and sodium nitrite solutions were carefully measured in the apparatus and the volume in the reaction chamber was carefully adjusted to the 4 cc. graduation before admitting the sample.

The rapidity of shaking was adjusted to 250 to 300 r.p.m., this being deemed sufficient to remove all liberated nitrogen. The temperature of the room was carefully controlled and the reagents were kept adjusted to this temperature. The latter precaution is of the utmost importance as will be shown subsequently. The writers are satisfied that the temperatures recorded represent those of the reaction mixtures to $\pm 0.3°C$.

The details of the Van Slyke procedure are too well known to require further comment. The various periods of the reaction were timed with the greatest possible precision and the liberated

TABLE 2

The amount of reaction is independent of the ammonium salt used

Temperature 25°C. Pressure 735 mm. Shaking three minutes. Sample = 1 cc.

AMMONIUM SALT USED	CONCENTRATION (NITROGEN PER CUBIC CENTIMETER	NITROGEN EVOLVED	PER CENT TOTAL NITROGEN EVOLVED
	mgm.	*mgm.*	
Acetate	5.0	1.630	32.6
Butyrate	5 0	1.680	33.6
Chloride	5.0	1.679	33.6
Acetate	2.5	0.825	33.0
Butyrate	2.5	0.816	32.6
*Gelatin + butyrate	2.5	0.865	34.6

* Five per cent Nutrient gelatin (Difco) + ammonium butyrate (2.5 mgm. nitrogen per cubic centimeter) gave the following:

Average corr. cc. nitrogen from 1 cc. above 2.24
Average corr. cc. nitrogen from 1 cc. gelatin control 0.63
From butyrate ..... 1.61 cc. = 0.865 mgm.

TABLE 3

Data to determine the order of the ammonia reaction

Temperature 25.5°C. Pressure 745 mm. Sample = 1 cc. Concentration = 5 mgm. nitrogen per 1 cc.

TIME	NITROGEN EVOLVED		PER CENT OF TOTAL NITROGEN EVOLVED
minutes	*corr. cc.*	*mgm.*	
2	2.05	1.02	20.4
3	3.10	1.52	30.4
4	4.10	2.13	42.6
5	4.70	2.46	49.2
8	5.90	3.13	62.6
10	6.30	3.36	67.2
15	7 80	4.15	80.3

gases were removed with the utmost celerity after the desired reaction time had elapsed. These details are necessary to obtain concordant results with ammonium salts, although such

precautions are obviously unnecessary with amino acids where the reaction proceeds to completion before three minutes (the usual time of shaking) has elapsed.

The factors which characterize any chemical reaction proceeding with a measurable velocity are: (1) the order of the reaction, (2) the temperature coefficient, (3) the concentration of the reactants.

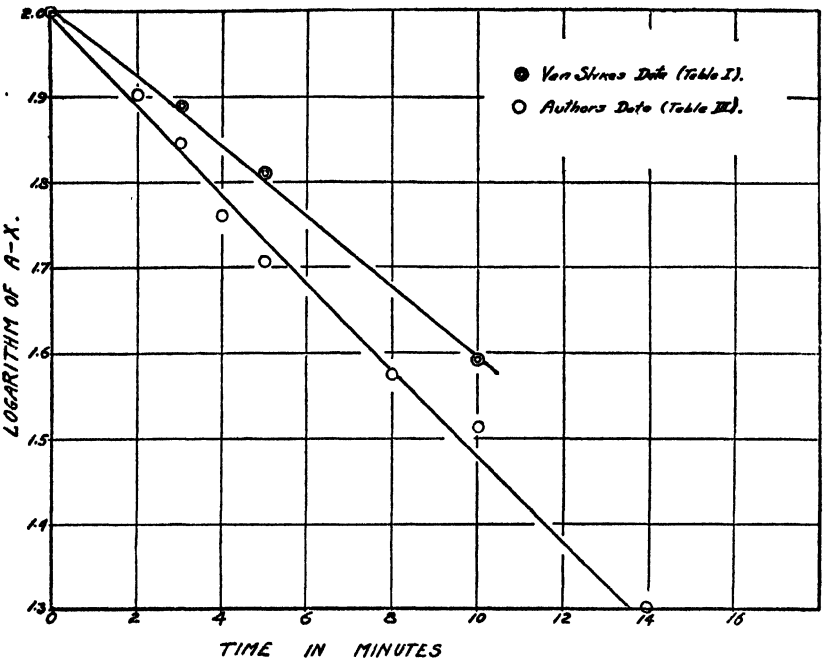

CHART 1. THE ORDER OF THE REACTION

It became necessary first to determine whether various ammonium salts reacted to the same degree. Table 2 shows the results obtained with a number of salt solutions.

It is evident from table 2 that the amount of nitrogen evolved is, within the limits of experimental error, independent of the salt used and is not influenced to any appreciable extent by the presence of even 5 per cent of gelatin.

The data given in table 3 were obtained with ammonium acetate to determine the order of the reaction.

If the reaction is monomolecular the logarithm of the concentration of the ammonium salt remaining in the reaction chamber at the end of time t plotted against time will be a straight line. The plots of the data in table 3 as well as the data of Van Slyke (table 1) are given in chart 1.

Both sets of results plotted in this way give straight lines. Their different positions on the chart are due to different reaction velocities resulting from different temperatures and different concentrations[1] of reagents in the reaction mixture.

TABLE 4

Calculation of velocity constant

t	x	$a - x$	$\frac{a}{a-x}$	$\log \frac{a}{a-x}$	$\frac{2.303}{t}$	k
			$a = 100$			
2	20.4	79.6	1.25	0.097	1.150	0.108
3	30.4	69.6	1.44	0.158	0.770	0.122
4	42.6	57.4	1.74	0.241	0.575	0.139
5	49.2	50.8	1.97	0.294	0.462	0 138
8	62.8	37.4	2.67	0.427	0.288	0.123
10	67.2	32.8	3.05	0.484	0.230	0.111
15	80.3	19.7	5.06	0.704	0 154	0.108
			Van Slyke's data (table 1) $a = 28.02$			
3	24.5	75.5	1.32	0 121	0.770	0.0875
5	36.3	63.7	1.57	0.196	0.462	0.0905
10	62.1	37.9	2 64	0.422	0.230	0.0970

Table 4 shows the velocity constants (K) calculated from the monomolecular reaction law[2] for the data in tables 1 and 3. For the sake of uniformity the results are calculated in percentages.

[1] Van Slyke used the macro-apparatus with 10 cc. of sample. This corresponds to the concentration obtained if a 2 cc. sample is used in the micro-apparatus. The results in table 3 were obtained from 1 cc. samples.

[2] The velocity of a monomolecular reaction is given by the equation

$$k = \frac{1}{t} \log_e \frac{a}{a - x}$$

where k is the velocity constant; t is the interval of time during which the reaction takes place, a is the initial concentration and x is the change in concentration, i.e., the amount of nitrogen evolved in the time t. For further details consult any text book of physical chemistry.

The values thus obtained for k are probably as constant as could be expected when one considers the experimental difficulties involved.

Since the reaction is undoubtedly monomolecular it follows that the rate of reaction is independent of the concentration of the ammonium salt, i.e., the per cent of the total nitrogen evolved in a definite interval will be the same regardless of the initial concentration of the ammonium salt.

TABLE 5

The amount of nitrogen evolved is independent of the concentration of ammonium salt

$t = 25°C$. Sample = 1 cc. Shaking = three minutes.

CONCENTRATION (NITROGEN PER CUBIC CENTIMETER)	NITROGEN EVOLVED	
mgm.	*mgm.*	*per cent*
1.0	0.32	32.0
2.5	0.82	32.8
5.0	1.68	33 6

These results show that within the limits of the probable experimental error the above statement is correct and may be taken as additional evidence that the reaction follows the monomolecular law.

TABLE 6

Per cent of nitrogen evolved between 20°C. and 35°C.

Shaking = three minutes. Concentration = 5 mgm. per cubic centimeter. Sample = 1 cc.

AMMONIUM SALT USED	NUMBER OF DETERMINATIONS	TEMPERATURE	AVERAGE AMOUNT OF NITROGEN EVOLVED	
			mgm.	*per cent*
Acetate	3	22.5	1.255	25.1
	2	23.5	1.405	28.1
	2	25.5	1.565	31.3
	1	27.0	1.940	38.8
	1	29.5	2.140	42.8
	2	32.5	2.725	54.5
Chloride	1	22.5	1.410	28.2
	1	25.5	1.720	34.4
	1	27.0	1.920	38.4
	1	32.0	2.580	51.6

The rate of a chemical reaction is usually doubled or tripled with each 10° rise in temperature. The temperature coefficient for this reaction between 20°C. and 30°C. may be stated thus

$$C = \frac{k_{30}}{k_{20}}$$

k being the velocity constant at the specified temperature. For the actual determination of C it was considered that the most

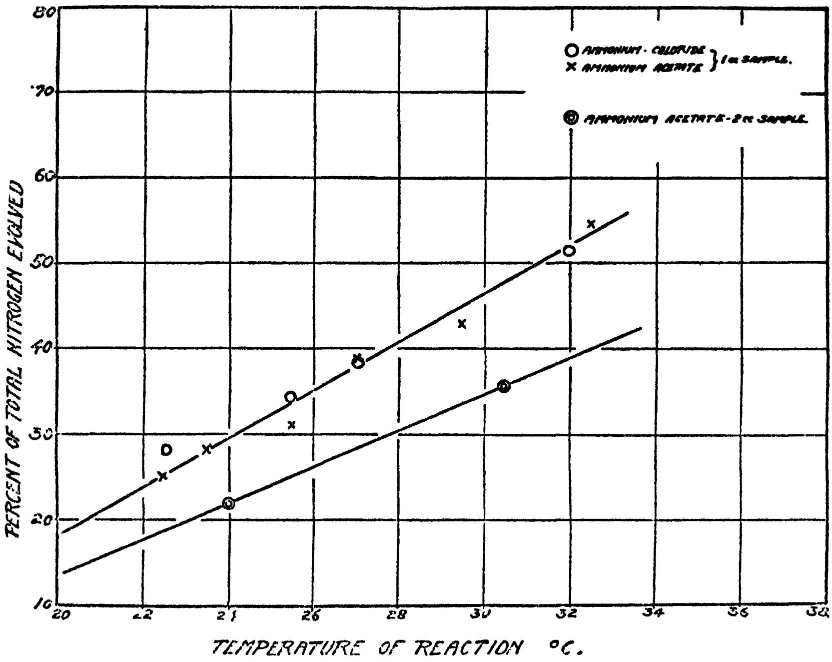

CHART 2. THE EFFECT OF TEMPERATURE AND SIZE OF SAMPLE ON THE AMOUNT OF AMMONIA NITROGEN EVOLVE

reliable result could be obtained by observing the amounts of nitrogen evolved in three minutes at various temperatures over this range.

Two sets of observations were made using two different ammonium salts. The results are recorded in table 6.

The results in table 6 are plotted in chart 2. The percentages of nitrogen evolved as taken from the curve at 20° and 30°C.

are 18 and 46 per cent respectively. This gives $k_{20} = 0.067$ and $k_{30} = 0.205$ and the temperature coefficient (20°C. – 30°C.).

$$C = 3.0$$

It is to be expected that the concentrations of the acetic acid and of the sodium nitrite will affect the reaction velocity. If this is the case, proportionate amounts of nitrogen will not be obtained from 1 cc. and 2 cc. samples. It is to be expected that the larger sample will give relatively less nitrogen. Table 7 shows the amounts of nitrogen obtained at two different temperatures from 2 cc. samples.

TABLE 7

Nitrogen evolved from 2 cc. samples of ammonium acetate

Sample = 2 cc. Shaking = three minutes. Concentration = 10 mgm. per 2 cc.

TEMPERATURE	NITROGEN EVOLVED		
	Milligrams	Average	Average per cent
°C.			
24.0	2.11 2.27 2.22 2.22	2.20	22.0
30.5	3.53 3.57 3.50 3.65 3.60	3.57	35.7

The values in table 7 are plotted in chart 2 and make possible a direct comparison with the results obtained with 1 cc. samples. The temperature coefficient (20°C. – 30°C.) calculated from this curve is 2.9 which is in good agreement with the value 3.0 obtained with 1 cc. samples. This speaks well for the consistency of the results.

DISCUSSION

It should be pointed out that the experimental results herein detailed are as close approximations as can be obtained with

the usual Van Slyke procedure. While greater precision could doubtless be obtained by introducing obvious refinements, it was rather the purpose of this work to observe the ammonium reaction under conditions usually employed.

The results show definitely that the reaction has a high temperature coefficient. This fact alone is sufficient to explain the failure of some investigators to obtain checks since in our experience the laboratory temperature may fluctuate 4° to 5°C. in a short period of time. Chart 2 shows that 18 per cent of the total ammonia is evolved in three minutes at 20°C., 32 per cent at 25°C. and 46 per cent at 30°C. Another factor which may lead to poor checks is failure to time the shaking accurately when ammonia is present. A smooth curve drawn from the data in Table 4 indicates that three and one-half minutes shaking yields 4 per cent more of the total ammonia than does a three minute shaking. This amounts to a very considerable error in many investigations, especially those studies of bacterial metabolism in which the ammonia nitrogen may be equal to or greater than the amino nitrogen.

Another factor which may give discordant results is the failure to measure accurately the reagents used, either initially, or in the deaminizing chamber at the time of admitting the sample. The effect of variation in the amount of sample is shown graphically in chart 2. In three minutes shaking at 25°C. the 2 cc. sample yields 24 per cent of its ammonia nitrogen as compared with 33 per cent for a 1 cc. sample. This difference is due to the dilution of the reagents by the larger sample.

The curves presented in chart 2 afford a possible basis for correcting results obtained from samples of which the ammonia content is known. The writers feel, however, that such a procedure is of questionable value since in most cases it is practicable to remove ammonia by some suitable method before making the Van Slyke determination.

The following results are offered to show what may be expected from a correction of Van Slyke analyses of material high in both amino and ammonia nitrogen. The sample used was a composite of several twenty-day-old nutrient gelatine cultures of *C. flabelliferum*.

	mgm.
Ammonia N (Folin)	345
Amino N (Van Slyke):	
1. NH_3 not removed, 1 cc. sample, 27°C	485
2. NH_3 removed by K_2CO_3 and aeration	334
3. NH_3 removed by $Ca(OH)_2$ and evaporation	371
Amino N (1) corrected[3] for NH_3	354

These results show that the corrected amino nitrogen is midway between the figures obtained after the removal of ammonia by two different methods commonly used. They further indicate that a study should be made to determine the most suitable procedure for the removal of ammonia from bacterial cultures.

TABLE 8

ANALYSIS NUMBER	OBSERVER	CORR. CC.	NITROGEN PER 100 CC.	DEVIATION FROM MEAN	
			mgm.	mgm.	per cent
1	WSS	5.36	142.0	−1.1	0.77
2	WSS	5.41	143.3	+0.2	0.14
3	WSS	5.36	142.0	−1.1	0.77
4	LBP	5.41	143.3	+0 2	0.14
5	LBP	5.41	143.3	+0.2	0.14
6	LBP	5.46	144.6	+1.5	1.05
Mean			143.1		
1	LBP	1.96	51.9	+0 54	1.05
2	LBP	1.94	51.4	+0.04	0.08
3	LBP	1 91	50.6	−0.76	1.48
4	LBP	1.98	52.4	+1.04	2.03
5	LBP	1.95	51.6	+0.24	0.47
6	WSS	1.95	51.6	+0.24	0.47
7	WSS	1.93	51.1	−0.26	0.51
8	WSS	1.90	50.3	−1.06	2.06
9	WSS	1.92	50.8	−0.44	0.87
10	WSS	1.96	51.9	+0 54	1.05
Mean			51.36		

Despite the difficulties experienced by Lamson and others which have caused some to discard the method, the writer's

[3] At 27°C. (chart 2) 38 per cent of the total ammonia nitrogen is evolved. This amounts to 131 mg. giving a corrected amino nitrogen of 485 − 131 = 354 mgm.

results, after the removal of ammonia, have been entirely satisfactory. The results of two series of consecutive analyses on two different samples of twenty-day-old *C. sporogenes* cultures in nutrient gelatin are presented in table 8.

As may be seen in table 8 the maximum deviation from the mean in two series of analyses by two different analysts was 2.06 per cent. This error is almost certainly within the limits of the accuracy of the measurements of the 2 cc. sample. Lamson's maximum deviations from the mean as computed from his Tables II and III, which are comparable in amino nitrogen content to the latter part of table 8, are 7.2 and 9.4 per cent respectively. The cause for these greater deviations is presumably due to his failure to remove ammonia.

SUMMARY

1. Data have been presented to show; the order of reaction, the temperature coefficient and the effect of dilution in the ammonia decomposition in the Van Slyke amino nitrogen procedure.

2. It has also been demonstrated that by removing ammonia satisfactory checks can *consistently* be obtained for amino nitrogen.

3. Results obtained without the removal of ammonia are too high by 18 to 50 per cent of the total ammonia nitrogen present depending upon the temperature and the size of the sample used. This may readily cause amino nitrogen values to be 50 per cent too high in cases where the ammonia nitrogen is equal to or greater than the amino nitrogen.

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THE POSSIBILITIES OF THE CONDUCTIVITY METHOD AS APPLIED TO STUDIES OF BACTERIAL METABOLISM

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Wider application is constantly being made of physical methods by workers in the biological field. Such methods are often easy to apply and enable a large number of determinations to be made in a minimum of time. Still wider applications of such methods would doubtless be possible if adequate interpretations of the results were available. Electrical conductivity is a simple and precise determination which has proved a valuable tool in many branches of science but which has not found any wide application in studies of bacterial metabolism. Oker-Blom used the method in the study of fermentation by *B. coli* and *B. typhosus* but failed to reach any very definite conclusions. Sturges and Rettger used the method in a study of the proteolytic activity of bacterial enzymes.

The purpose of this paper is to present a resume of conductivity changes as correlated with ammonia and amino acid changes in cultures of *C. sporogenes* and *C. flabelliferum* in several media. These results were chiefly incidental to a comparative study of the nitrogen metabolism of these organisms.

EXPERIMENTAL

The conductivity equipment consisted of Leeds and Northrop instruments as follows: (*a*) No. 4760 Wheatstone bridge, dial type, direct reading, range 1–100,000 ohms; (*b*) No. 2370-B a. c. galvanometer; (*c*) No. 9816 transformer. This apparatus operates on a 110 V. a. c. circuit and has a guaranteed accuracy of 0.5 per cent. The conductivity cell, shown in figure 1, was

constructed by the senior writer and found entirely satisfactory. The details of construction are apparent. The stout copper leads h-h dip into mercury cups fixed above the thermostat and enable the cell to be quickly and easily removed for filling, cleaning etc. The capacity of this particular cell was 7 cc. The cell constant, using thrice recrystallized potassium chloride in N/50 solution was found to be 2.205. This was of a magnitude to insure resistance readings of a convenient size with the materials used.

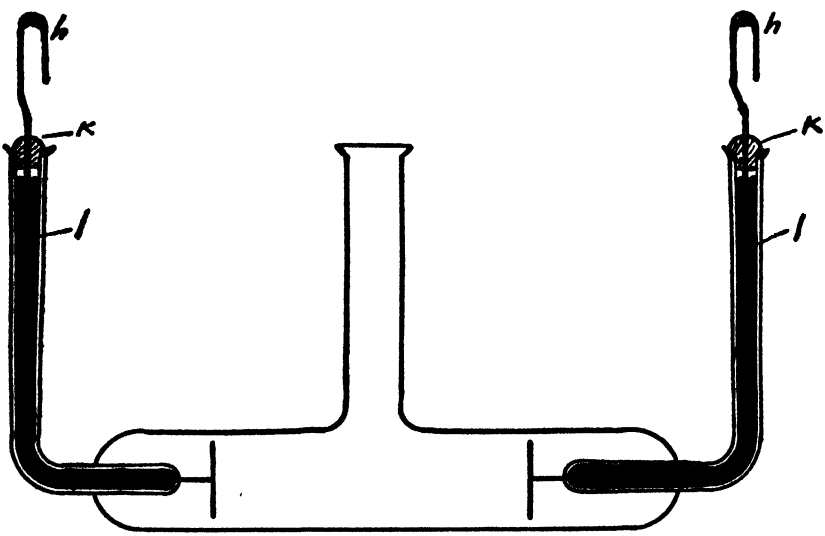

FIG. 1. THE CONDUCTIVITY CELL

hh, copper hooks; *kk*, DeKhotinsky cement seals; *ll*, mercury contacts to platinum electrodes.

The thermostat temperature was maintained at 30°C. during these determinations. It was found that thermal equilibrium was reached very quickly if the cultures were kept immersed in the thermostat water before filling the cell. Not more than five minutes was required for a determination, of the order of accuracy necessary in work of this character.

The media used were nutrient gelatin (Difco dehydrated), milk, and nutrient broth (pepton, 2 per cent). For each series five tubes of media were inoculated with each culture under

investigation. These were incubated at 35°C. in an atmosphere of hydrogen; and one tube was analyzed on the second, fourth, sixth, tenth, and twentieth day respectively. After the determination of the conductivity, the sample was used for the determination of ammonia (Folin), amino nitrogen (both Van Slyke and Sörensen (Brown, 1923) and electrometric pH.

After critically examining the large amount of data which became available in the course of these studies the writers concluded that a plot of conductivity change against change in ammonia and against the change in Sörensen values (amino N + ammonia N) would be most valuable in interpreting the results.

TABLE 1

Conductivity and nitrogen metabolism of C. flabelliferum in gelatin media

CULTURE	AGE	FORMOL TITRATION		AMMONIA NITROGEN		CONDUCTIVITY	
		Nitrogen per 100 cc.	Change	Nitrogen per 100 cc.	Change	Ohms^{-1} $\times 10^{3}$	Change
	days	*mgm.*		*mgm.*			
Control	0	60		10		3 5	
C. flabelliferum, 1	2	143	83	60	50	6.2	2 7
	4	251	191	140	130	10.2	6.7
	6	286	226	179	169	11 8	8.3
	10	455	395	255	245	15.8	12.3
	20	792	732	424	414	23 6	20.1

In obtaining the values plotted, the initial values; i.e., the conductivity ammonia, etc., of the sterile media, were subtracted from each subsequent determination on the cultures.

Table 1 shows a typical series of results obtained with a 10 per cent nutrient gelatin culture of *C. flabelliferum.*

Chart 1 shows the data obtained on two series of determinations using two different strains each of *C. flabelliferum* and *C. sporogenes* in gelatin media. No differentiation has been made between the individual strains of *C. flabelliferum* and C. *sporogenes*, the former being designated by crosses and the latter by circles. There can be no question that change in ammonia is proportional to the change in conductivity over the wide range

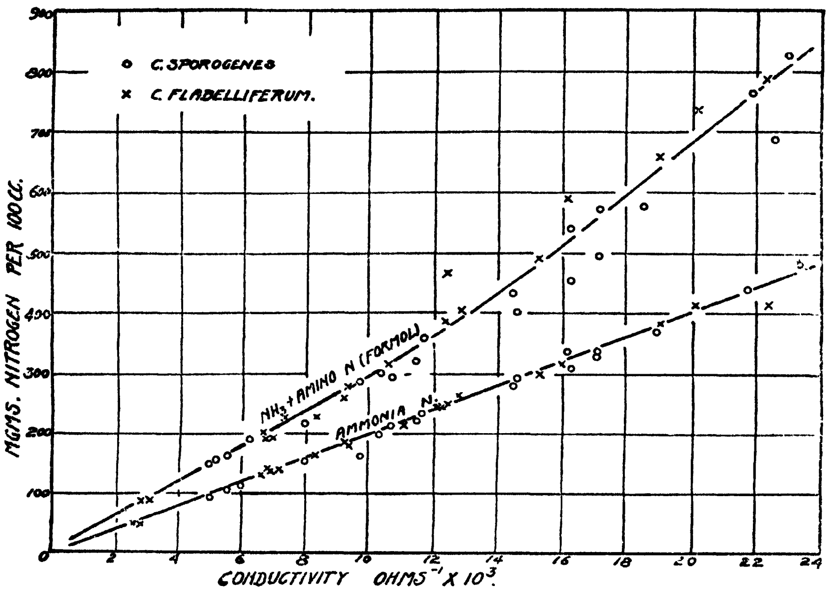

CHART 1. THE VARIATION IN AMMONIA AND FORMOL TITRATION WITH CHANGE IN CONDUCTIVITY IN GELATIN

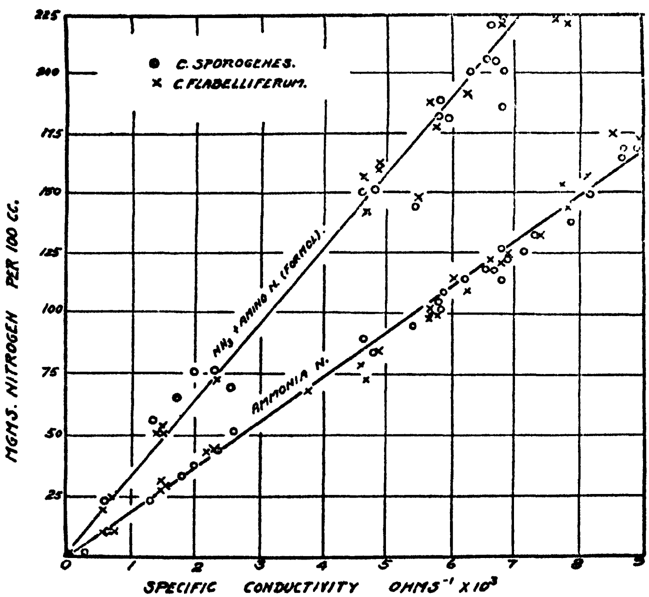

CHART 2. THE VARIATION IN AMMONIA AND FORMOL TITRATION WITH CHANGE IN CONDUCTIVITY IN MILK

studied. If the formol titration (the sum of amino and ammonia nitrogen) be taken as an index of proteolytic action, this chart further shows that change in conductivity represents fairly accurately the various degrees of proteolysis.

Chart 2 was plotted in a similar way from data obtained on three series of determinations using the same organisms in milk media. Here again it will be noted that change in conductivity

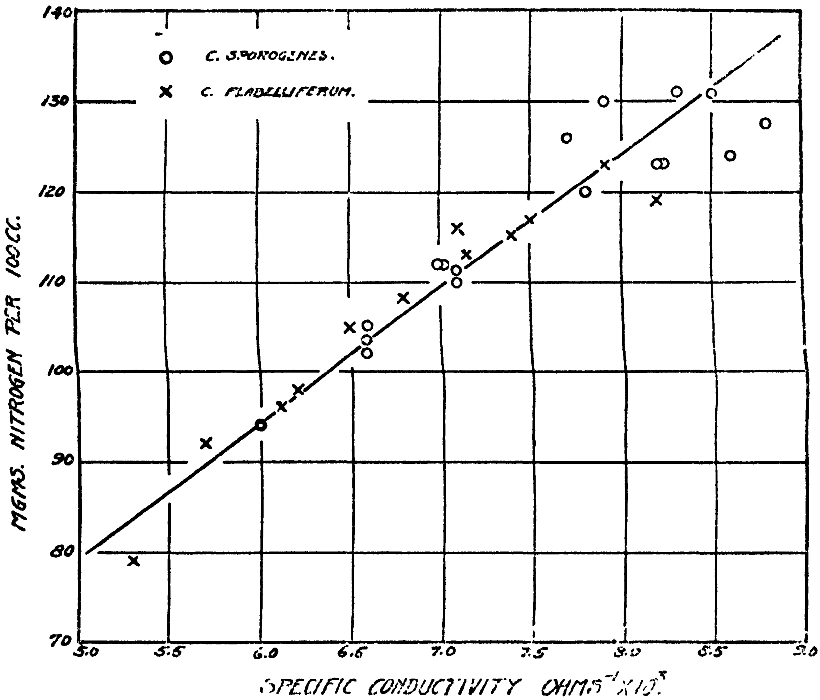

CHART 3. THE VARIATION IN AMMONIA NITROGEN WITH CHANGE IN CONDUCTIVITY IN PEPTON

is proportional to change in ammonia and also that change in degree of proteolysis follows change in conductivity.

The data on pepton media were not so satisfactory since a large amount of the total change occurred in the first two days. For the sake of completeness the ammonia data are presented in chart 3. It can be seen that here, also, the ammonia change is proportional to the change in conductivity. The formol values

TABLE 2

Comparison of ammonia nitrogen (Folin) with that calculated from change in specific conductivity

Eight strains of *C. sporogenes* in nutrient gelatin

CULTURE NUMBER	AGE	AMMONIA NITROGEN PER 100 cc.	SPECIFIC CONDUCTIVITY OBSERVED	$OHMS^{-1} \times 10^3$ CHANGE	CHANGE IN AMMONIA: Observed	CHANGE IN AMMONIA: Calculated	DIFFERENCE	
	days	*mgm.*					*mgm.*	*per cent*
Control	0	6	3.5					
W-66	4	163	11.6	8 1	157	158	+1	0.6
	6	161	11.8	8.3	155	162	+7	4.5
	10	283	17.4	13.9	177	171	+6	3.4
	20	413	23.7	20.2	407	394	−13	3.2
W-88	2	126	9.7	6.2	120	121	+1	0.8
	4	185	12 6	9 1	179	175	−4	2 2
	6	249	16.7	13.2	243	257	+14	5.8
	10	359	20 4	16.9	353	329	−24	6.8
	20	460	27.4	23.9	454	466	+12	2.6
W-84	2	138	10 4	6.9	132	134	+2	1.5
	4	226	15 0	11.5	220	224	+4	1 8
	6	256	16 9	13 4	250	261	+11	4.4
	10	350	20.1	16.6	344	324	−20	5.8
	20	460	24 5	21.0	454	409	−45	9.9
W-52	2	109	8.8	5.3	103	103	0	0.0
	4	188	12.8	9 3	182	181	−1	0.6
	6	198	13.1	9.6	192	187	−5	2 6
	10	317	18.4	14.9	311	291	−20	6.4
	20	440	24.0	20.5	434	400	−34	7.6
H-52	2	106	8.8	5.3	100	103	+3	3 0
	4	185	13 0	9.5	179	185	+6	3.3
	6	276	17 6	14.0	270	273	+3	1.1
	20	423	23.7	20.2	417	394	−23	5.5
H-54	2	168	12.0	8.5	162	165	+3	1.8
	4	223	15 2	11.7	217	228	+11	5.1
	6	227	15.1	11.6	221	226	+5	2.2
	10	290	17.6	14.2	284	277	−7	2.4
	20	424	22.5	19.0	418	371	−47	11.2

TABLE 2—*Continued*

CULTURE NUMBER	AGE	AMMONIA NITROGEN PER 100 CC.	SPECIFIC CONDUCTIVITY OBSERVED	OHMS^{-1} × 10^3 CHANGE	CHANGE IN AMMONIA Observed	CHANGE IN AMMONIA Calculated	DIFFERENCE	
	days	*mgm.*					*mgm.*	*per cent*
H-48	2	132	10.2	6.7	126	130	+4	3 2
	4	165	12.5	9.0	159	175	+16	10.1
	6	193	13.5	10.0	187	195	+8	4 3
	10	286	17.4	13.9	280	271	−9	3.2
	20	436	23.7	20.2	430	394	−36	8.4
H-74	2	119	9 6	6.1	113	119	+6	5 8
	4	184	12.9	9.5	178	185	+7	3 9
	6	213	14.6	11.1	207	216	+9	4 3
	10	283	17 3	13 8	277	269	−8	2 9
	20	424	22 7	19 2	418	374	−44	10 5

are not shown on the chart since they were only a trifle larger than the ammonia values and could not be plotted without causing confusion.

Charts 1 to 3 show clearly that change in ammonia is proportional to change in conductivity. A further comparison of a large number of strains of *C. sporogenes* and *C. flabelliferum* on gelatin media, afforded an opportunity to give the conductivity method a rigid test. From the data presented in chart 1 it is possible to calculate a constant, for these particular organisms at least by which the change in specific conductivity must be multiplied in order to give the change in ammonia nitrogen in milligrams per 100 cc. This constant[1] is 1.95×10^4 for gelatin media.

Table 2 shows the results obtained for ammonia as calculated by the conductivity changes compared with the results obtained by the Folin method, using a number of strains of *C. sporogenes* in gelatin media.

[1] This is easily calculated by determining the slope in the equation of the curve in chart 1. The equation for a straight line is $y = mx + b$. Since the line pass through the origin $b = o$. Therefore, $m = y/x$ and it is only necessary to make a number of readings of x and y and perform the necessary division to obtain the constant.

TABLE 3

Comparison of ammonia nitrogen (Folin) with that calculated from change in specific conductivity

Eight strains of *C. flabelliferum* in nutrient gelatin

CULTURE NUMBER	AGE	AMMONIA NITROGEN PER 100 CC.	SPECIFIC CONDUCTIVITY OBSERVED	OHMS^{-1} × 10^3 CHANGE	CHANGE IN AMMONIA		DIFFERENCE	
					Observed	Calculated		
	days	*mgm.*					*mgm.*	*per cent*
Control	0	11	3.5					
F-1	2	93	7.9	4.4	82	86	+4	4.9
	4	134	10.5	7.0	123	136	+13	10.5
	6	185	11.8	8.3	174	162	−12	6.9
	20	368	20.5	17.0	357	332	−25	7.0
F-2	2	161	11.6	8.1	150	158	+8	5.3
	4	213	14.7	11.2	202	218	+16	7.8
	6	299	17.0	13.5	288	263	−25	8.7
	10	363	21.2	17.7	352	345	−7	2.0
	20	444	24.9	21.4	433	418	−15	3.5
F-3	2	137	10.2	6.7	126	131	+5	4.0
	4	208	14.0	10.5	197	205	+8	4.1
	10	313	20.1	16.6	302	324	+22	7.3
	20	403	21.9	18.4	392	359	−33	6.7
F-4	2	244	14.1	10.6	233	207	−26	11.1
	4	287	17.7	14.2	276	276	0	0.0
	6	373	20.8	17.3	362	338	−24	6.7
	10	450	26.2	22.7	439	443	+4	0.9
	20	528	28.5	25.0	517	488	−29	5.6
F-5	2	140	10.3	6.8	129	133	+4	3.1
	4	196	13.3	9.8	185	191	+6	3.2
	10	267	21.4	17.9	356	350	−6	1.7
	20	423	23.4	19.9	412	388	−24	5.0
F-6	2	161	11.2	7.7	150	150	0	0.0
	4	223	14.3	10.8	212	211	−1	0.5
	6	447	23.7	20.2	436	394	−42	9.6
	10	382	21.8	18.3	371	357	−14	3.8
	20	442	23.8	20.3	431	393	−38	8.8

TABLE 3—*Continued*

CULTURE NUMBER	AGE	AMMONIA NITROGEN PER 100 CC.	SPECIFIC CONDUCTIVITY OBSERVED	OHMS^{-1} × 10^{3} CHANGE	CHANGE IN AMMONIA		DIFFERENCE	
					Observed	Calculated		
	days	*mgm.*					*mgm.*	*per cent*
F-7	2	206	13.8	10.3	195	202	+7	3.6
	4	228	15.7	12.2	217	238	+21	9.7
	6	474	24.9	21.4	463	418	−45	9.7
	10	454	25.1	21.6	443	424	−19	4.3
	20	495	26.6	23.1	484	452	−32	6.6
F-8	2	175	11.9	8.4	164	164	0	0.0
	4	262	16.7	13.2	251	258	+7	2.8
	10	442	24.0	20.5	431	400	−31	7.2

Similar results with several strains of *C. flabelliferum* are given in table 3.

These results indicate that the factor derived from chart 1, using two strains each of *C. flabelliferum* and *C. sporogenes*, can be applied successfully for the calculation of ammonia changes from conductivity changes in gelatin cultures of many strains of these organisms. The maximum difference between the calculated and the observed ammonia change was 11 per cent and the difference was usually very much less than this. A close inspection of the tables shows that the higher ammonia values almost invariably show a lower calculated ammonia (from conductivity) than the observed value. This fact suggests that the ammonia-conductivity curve is not exactly a straight line but has a slight upward curvature such as is shown by the conductivity curves for pure ammonium salts in chart 4. If such is the case the constant already derived would not be exactly correct for all values. It would be possible to derive a more exact relation between conductivity and ammonia changes but it scarcely seems worth while at the present time.

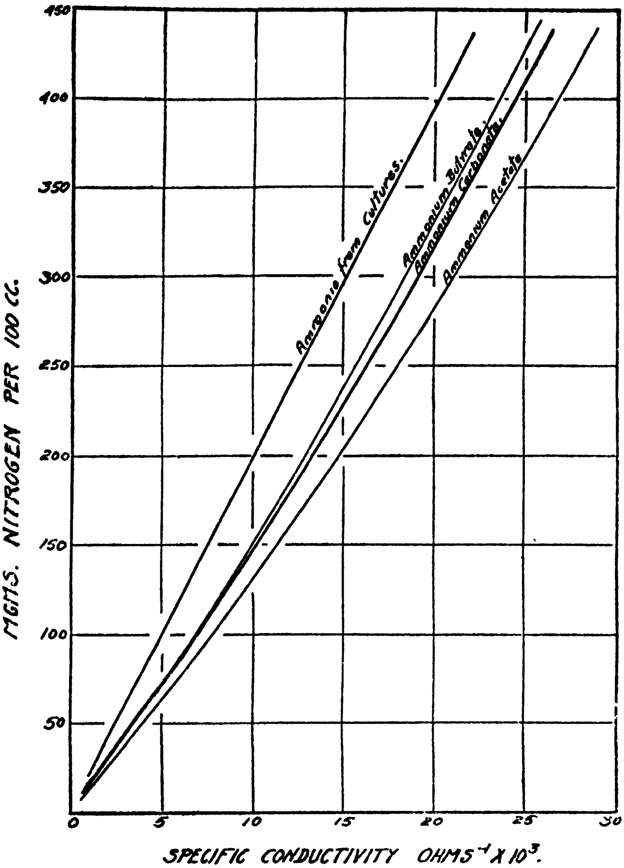

CHART 4. THE CONDUCTANCE OF AMMONIA IN CULTURES COMPARED WITH THE CONDUCTANCE OF VARIOUS AMMONIUM SALTS

DISCUSSION

The results herein presented seem to permit of the conclusion that conductivity may be used as a fairly exact measure of the ammonia produced by *C. sporogenes* and *C. flabelliferum* in several different media. It has further been demonstrated that the course of proteolysis as indicated by the formol titrations follows the change in conductivity rather closely. Since the ammonia nitrogen changes are so closely related to changes in specific

conductivity it became interesting to determine the specific conductivities of some ammonium salts which might well be present in gelatin cultures of these organisms. Measurements of the acetate, butyrate and carbonate at 30°C. are presented in chart 4. These are contrasted with the conductivity-ammonia changes observed in gelatin cultures of *C. flabelliferum* and *C. sporogenes*. These curves show that equivalent amounts of ammonium nitrogen give higher conductivities in the case of the ammonium salts than in the case of the gelatin cultures. There are many factors which have to be considered in attempting to explain this point. One obvious explanation would be that the treatment for the liberation of ammonia in the Folin method removes nitrogen which is not in the form of ammonium salts. Amide nitrogen might also conceivably be a source of this excess nitrogen. Again, ammonium salts of acids of lower conductivity than butyric might be present. Certainly it appears from the data in Chart IV that aspiration with sodium carbonate and oxalate is sufficient to remove all the ammonium in the form of ammonium salts. Any more drastic method proposed as being more efficient for an ammonia determination (Wagner, 1924) should be subjected to a very critical examination before being adopted. It might be expected that the nitrogen present in the form of amino acids, polypeptides and peptones would contribute materially to the conductivity. However, Bayliss in his study of the causes of conductivity changes in tryptic digests of gelatin presents data from which it may be concluded that the conductivity from these sources could not possibly account for much more than 5 per cent of the changes observed by the writers.

In any measurements of conductivity the pH change of the solution must be taken into consideration. The limits of pH in all the cultures studied were 5.5 and 8.0. It can be shown by a simple calculation that the specific conductivity due to the $(H+)$ at pH 5.5 is approximately 0.001×10^{-3} and that due to the (OH^-) at pH 8.0 is 0.0001×10^{-3}. These values are negligible in comparison with the observed changes.

An argument in favor of the conductivity method is the fact that in this measurement small changes in ammonia give large

changes in the resistance, and consequently in conductivity. For instance, when the ammonia nitrogen is increased by 400 mgm. the resistance in the cell used fell from 630 ohms to 110 ohms.

The writers recognize that the results presented may not be applicable to all cultures of proteolytic organisms. It has not been possible to undertake as extensive a study as would be necessary to establish the method on a satisfactory basis for a large number of organisms. There is no apparent reason why consistent results might not be obtained with any proteolytic culture. It is to be hoped that someone will have an opportunity to extend the scope of these observations, since the application of a simple method of this type can not fail to be of great value in studies of bacterial metabolism.

SUMMARY

1. The conductivity method has been suggested as a convenient means of following the rate of proteolysis in bacterial cultures.

2. It has been applied to a comparative study of several strains of *C. flabelliferum* and *C. sporogenes* in milk, nutrient gelatin, and pepton media.

3. The results establish the fact that in these media the change in conductivity is closely proportional to the change in ammonia, and follows rather closely the change in formol titration.

4. Data and calculations have been presented which show that the conductivity changes observed can be entirely accounted for by the ammonia found (Folin) if it is assumed to be present in the form of ammonium salts.

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FURTHER ISOLATIONS OF CLOSTRIDIUM FLABELLIFERUM WITH A COMPARISON OF THE NITROGEN METABOLISM OF SEVERAL STRAINS OF C. FLABELLIFERUM AND C. SPOROGENES

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A preliminary report has already been presented (1924) of an unusual type of putrefactive, anaerobic, clostridium. The most striking characteristic of this organism is the peculiar morphology of the sporangia, the vegetative portion of which invariably frays out longitudinally into innumerable fibers. This distinctive fan shaped appearance of the vegetative portion of the sporangium has suggested the name *C. flabelliferum*.

In the large number of isolations of putrefactive anaerobes made in this laboratory in the past four years, no organism similar to the culture originally isolated in 1921 was ever encountered until the summer of 1924. At this time a more or less routine examination of some materials from a western packing house revealed an abundance of anerobes having this striking morphology. This observation led to a limited bacteriological survey which disclosed the presence of putrefactive anaerobes of this type in salt (from Great Salt Lake), in sludge from salt leaching vats, and in certain specimens of some meats cured or partially cured with this salt. The dry salt in which a small amount of insoluble matter was present contained such a number of these putrefactive organisms that they were usually found in 1 cc. portions of a saturated solution and invariably in small portions of the flocculent sediment when inoculated into egg-meat media. The brush-like sporangia became visible after a few days incubation at 35°C. and in a few rare cases, where inoculation had been scanty, appeared to constitute a pure culture. Usually, however,

other organisms, both aerobic and anaerobic were present. In all cases extensive reisolations (in Veillon tubes, etc.) were resorted to. The absence of aerobes was, of course, assured by repeated failure to obtain growth aerobically on glucose agar slants using large inoculations. The absence of other anaerobes after repeated reisolations was established by exhaustive microscopic study. This is made possible by the property possessed by *C. flabelliferum* of retaining indefinitely in the spored state the frayed

FIG. 1. CLOSTRIDIUM FLABELLIFERUM. FOUR-DAY CULTURE IN EGG MEAT MEDIUM. MAGNIFICATION × 2000

vestigia of the vegetative cell. The presence of other anaerobic spores is readily detected among the characteristic sporangia of *C. flabelliferum*. *C. sporogenes* is especially easy to eliminate owing to its invariable early formation of free spores, which are never observed in pure cultures of *C. flabelliferum*.

Seven isolations of the new organism have been achieved which after repeated rigorous testing are considered to be pure cultures. These organisms have all been isolated from the materials mentioned above. In addition the presence of distinctive brush-

like sporangia has been noted in many other mixed cultures from the same sources.

Preliminary cultural tests wherein a comparison was made between two strains of *C. flabelliferum* (the original strain and a Salt Lake salt strain) and *C. sporogenes* (2 strains) has given some distinct differences. *C. flabelliferum* grown anaerobically in flasks of nutrient gelatin develops, sometimes within forty-eight hours, a few dense, viscid, gelatinous colonies adherent to the glass at the bottom of the flask. These colonies range from 1 to 4 mm. in diameter and have black centers with translucent borders. The turbidity of media offers another means of differentiation. *C. sporogenes* produces a heavy turbidity both in nutrient gelatin and in nutrient broth which persists even in old cultures. Cultures of *C. flabelliferum*, on the other hand, forty-eight hours or older, are invariably clear with the growth flocculated at the bottom of the tube. While the opportunity of observing the gelatin colonies has been limited to the first two strains, comparisons of turbidity have been made on all the cultures. This property was so striking that one could infallibly select the *C. flabelliferum* tubes from a collection of broth or gelatin cultures of the two organisms.

Since proteolysis is such an important consideration in the classification of anaerobes, it became of paramount importance to make a biochemical comparison of this property of the new organism with that of *C. sporogenes* which it closely resembles.

Various determinations have been proposed as quantitative criteria of proteolytic change. Among these may be noted ammonia, formol titration (amino + ammonia nitrogen), Van Slyke amino nitrogen, non-protein nitrogen, and the biuret test of Vernon. Considering the lack of agreement as to the relative merits of these determinations it appeared expedient to observe a number of these changes and to draw conclusions on the basis of all of the results.

As measures of proteolysis it was decided to follow changes in ammonia, formol titration, Van Slyke amino nitrogen, and conductivity.[1]

[1] The significance of conductivity change is discussed by the writers elsewhere.

By the proper choice of micro-methods it is possible to make all of these determinations in duplicate with 10 to 15 cc. of culture. This is a point of great convenience when so many observations are to be made using anaerobic procedure.

Ammonia was determined by a modified Folin-MacCallum method using 1 cc. samples with 1 cc. of a solution containing 10 per cent potassium carbonate and 15 per cent potassium oxalate. One cubic centimeter of kerosene prevented foaming. Ammonia was removed by aspiration into N/50 HCl and determined by titration of the excess acid using methyl red as an indicator.

Formol titrations were made on 1 cc. samples using J. H. Brown's (1923) modification B. of Sorensen's procedure. As an end point pH 8.0 was selected. Brown's precaution of titrating immediately after the addition of formol is to be emphasized for material of this nature in which CO_2 is undoubtedly present. For cultures in which ammonia and amino nitrogen is high, it becomes necessary to use a stronger sodium hydroxide solution (N/10 to N/5) for the final titration in order to avoid the dilution error. The writers consider that Brown's method is most satisfactory for use in bacteriological work. It was found possible to obtain checks to within 0.01 to 0.02 cc. consistently. A burette with 0.01 cc. graduations was, of course, used.

Van Slyke amino nitrogen determinations were made using the micro apparatus. Corrections for ammonia were made by the method suggested in another communication.

Conductivity was measured at 30°C. according to technique described by the writers in another paper.

The pH of all samples was determined electrometrically.

The rate of proteolysis was studied in nutrient gelatin (Bacto dehydrated), in beef extract broth (2 per cent Bacto Pepton), and in milk.

The cultures used were the eight isolations of *C. flabelliferum* previously mentioned (labelled F-1 to F-8) and nine strains of *C. sporogenes* obtained from the museum's collection and from Dr. I. Hall.

The media was tubed in 15 cc. portions and 0.2 cc. of a vigorous twenty-four-hour nutrient broth culture was used to inoculate

each tube. Media were always preheated to eliminate dissolved air. A sufficient number of tubes were inoculated with each

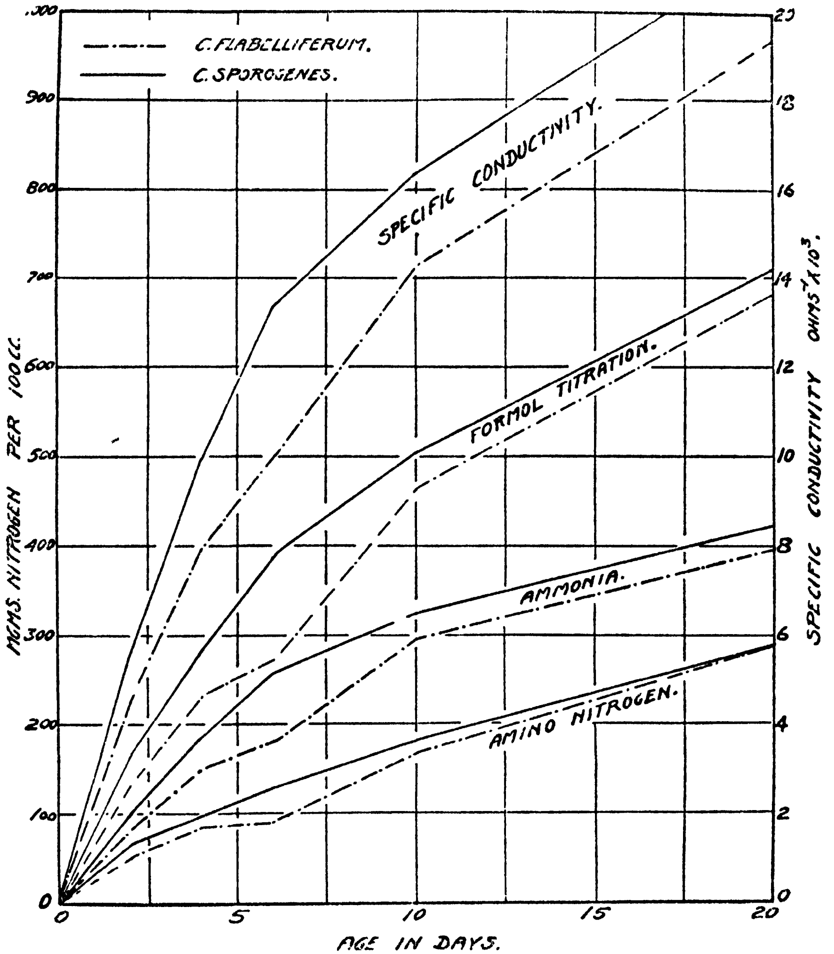

CHART 1. PROTEOLYTIC CHANGES IN NUTRIENT GELATIN MEDIA

Total nitrogen	1650 mgm. per 100 cc.
Formol nitrogen (initial)	60 mgm. per 100 cc.
Ammonia nitrogen (initial)	8 mgm. per 100 cc.
Specific conductivity (initial)	3.5×10^{-3} ohms.$^{-1}$

strain to furnish one tube for analysis on the second, fourth, sixth, tenth, and twentieth days respectively. Immediately

after inoculation the tubes were placed in suitable containers and anaerobic conditions were produced by evacuating and filling with hydrogen. To insure more complete removal of oxygen, the

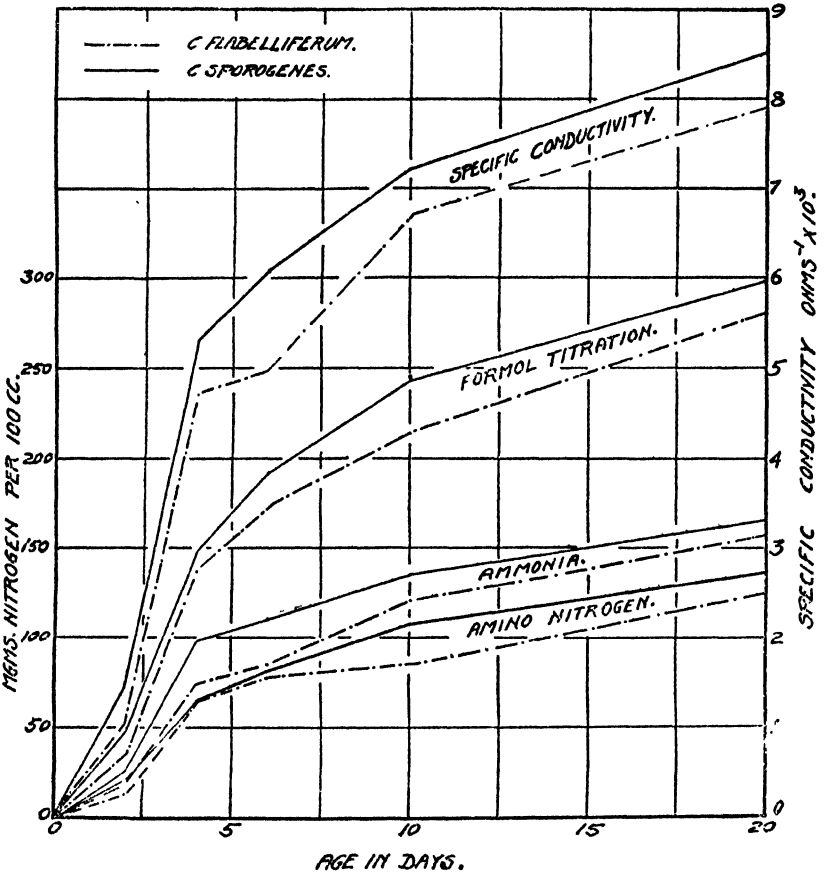

CHART 2. THE PROTEOLYTIC CHANGES IN MILK

Total nitrogen	515 mgm. per 100 cc.
Formol nitrogen (initial)	22 mgm. per 100 cc.
Ammonia nitrogen (initial	10 mgm. per 100 cc.
Specific conductivity (initial)	6.0×10^{-3} ohms.$^{-1}$

container was evacuated again and refilled with hydrogen. All cultures were incubated at 35°C. At the time of analysis each tube was tested for aerobic contamination.

Preliminary observations were made in three media (gelatin, milk and pepton) using two strains of each organism, designated as F-1, F-2; S-1 and H-52. Two or three series were run with

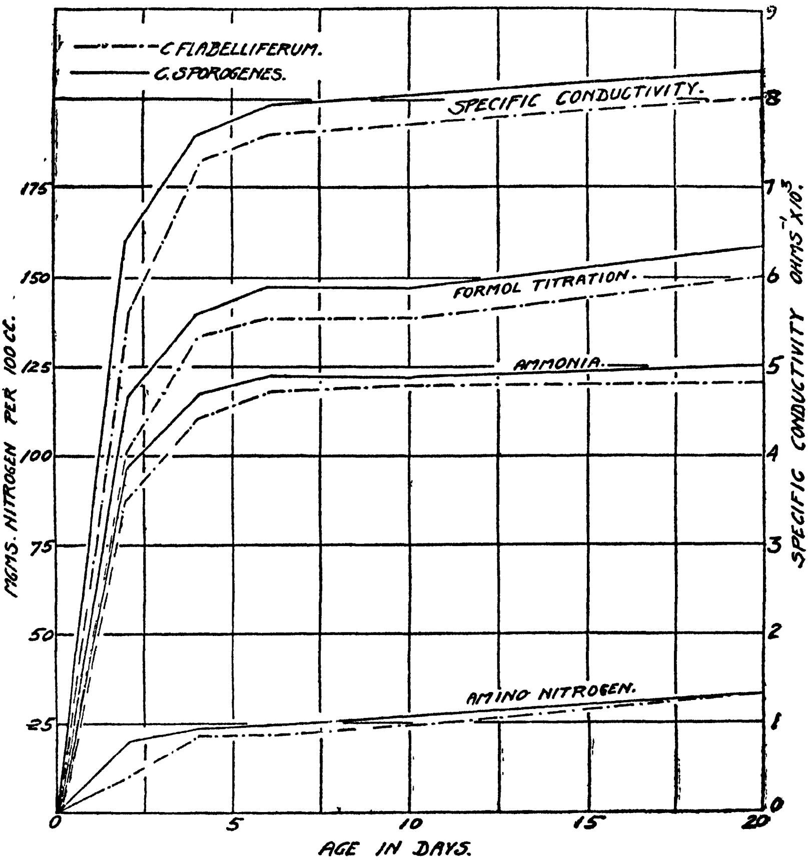

CHART 3. PROTEOLYTIC CHANGES IN NUTRIENT BROTH (2 PER CENT PEPTON)

Total nitrogen	319 mgm. per 100 cc.
Formol nitrogen (initial)	50 mgm. per 100 cc.
Ammonia nitrogen (initial)	10 mgm. per 100 cc.
Specific conductivity (initial)	5.0×10^{-3} ohms.$^{-1}$

ach media and the values obtained each day for the two strains f each organism were averaged. Such a mass of data results rom an extensive analytical study of this nature that it appears

neither necessary nor practicable to include the individual determinations. The writers consider that the material can be presented in the most intelligible form by graphical means. Charts 1, 2 and 3 summarize the results obtained. It will be noted that in each medium the changes in ammonia, formol titration,

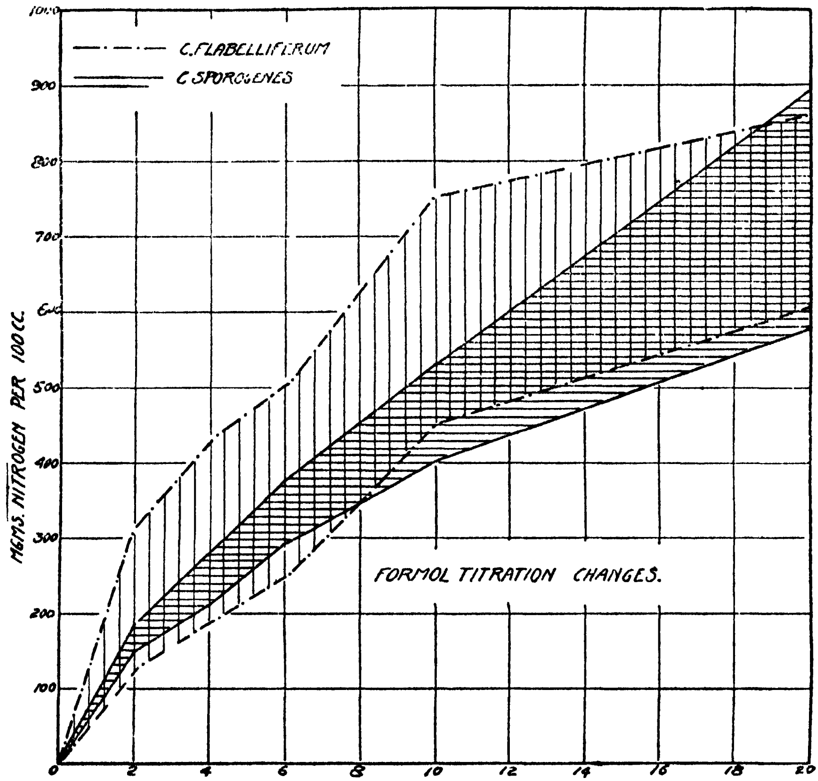

CHART 4. RANGE OF PROTEOLYTIC CHANGES BY C. FLABELLIFERUM AND C. SPOROGENES

Total nitrogen	1650 mgm. per 100 cc.
Formol nitrogen (initial)	60 mgm. per 100 cc.

amino nitrogen (formol minus ammonia), and conductivity with time indicate that, at least for these strains, *C. sporogenes* is slightly the more proteolytic.

The general similarity of the time curves for the two organisms for each property measured necessitates the conclusion that the

nature of proteolysis of both anaerobes is essentially the same in any one of the media used.

Throughout the course of these observations it was apparent that there were consistent differences in the proteolytic activities

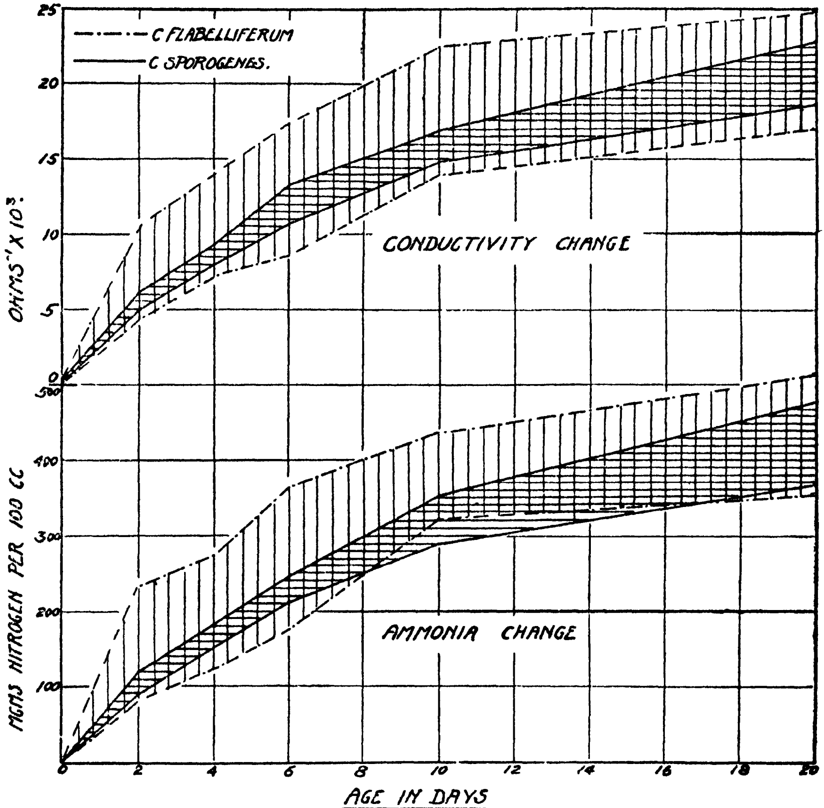

CHART 5. RANGE OF PROTEOLYTIC CHANGES BY C. FLABELLIFERUM AND C. SPOROGENES IN GELATIN

Total nitrogen.................................1650 mgm. per 100 cc.
Ammonia nitrogen (initial)........................ 10 mgm. per 100 cc.
Specific conductivity (initial)................... 3.5×10^{-3} ohms.$^{-1}$

of the two strains of the same organism. Thus H-52 was the more active sporogenes and F-2 the more active flabelliferum strain. In the light of this observation the rather slight differences between the average curves of the two organisms becomes

of questionable significance. It, therefore, seemed advisable to determine the range of the proteolytic activities which may be exhibited by different strains of each of these organisms. Gelatin was selected as the most suitable medium for a comparative study. A careful survey of the data obtained from the nine strains of *C. sporogenes* and the eight strains of *C. flabelliferum* showed S-1 and F-1 to be the least active while W-88 and F-4 were the most proteolytic.

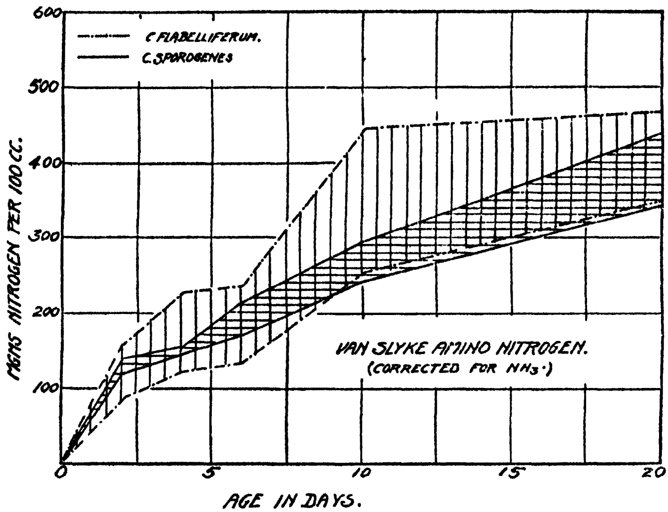

CHART 6. RANGE OF PROTEOLYTIC CHANGES BY C. FLABELLIFERUM AND C. SPOROGENES IN GELATIN

Total nitrogen.................................1650 mgm. per 100 cc.
Van Slyke amino nitrogen (initial).............. 63 mgm. per 100 cc.

In charts 4, 5 and 6 curves showing the changes in formol titration, ammonia, conductivity, and Van Slyke amino nitrogen with time for these four cultures are plotted. In all cases the lower solid line denotes culture S-1 which represents the least active sporogenes strain. The upper solid line denotes W-88, the most active sporogenes strain. The lower and upper broken lines denote F-1, and F-4; the least and the most active flabelliferum strains respectively. These charts show definitely that for the strains studied the zone of proteolysis of *C. sporogenes*

lies within the zone of proteolysis of *C. flabelliferum*.[2] The relationship of these zones suggests that, on the average, the flabelliferum cultures are more proteolytic than the sporogenes cultures.

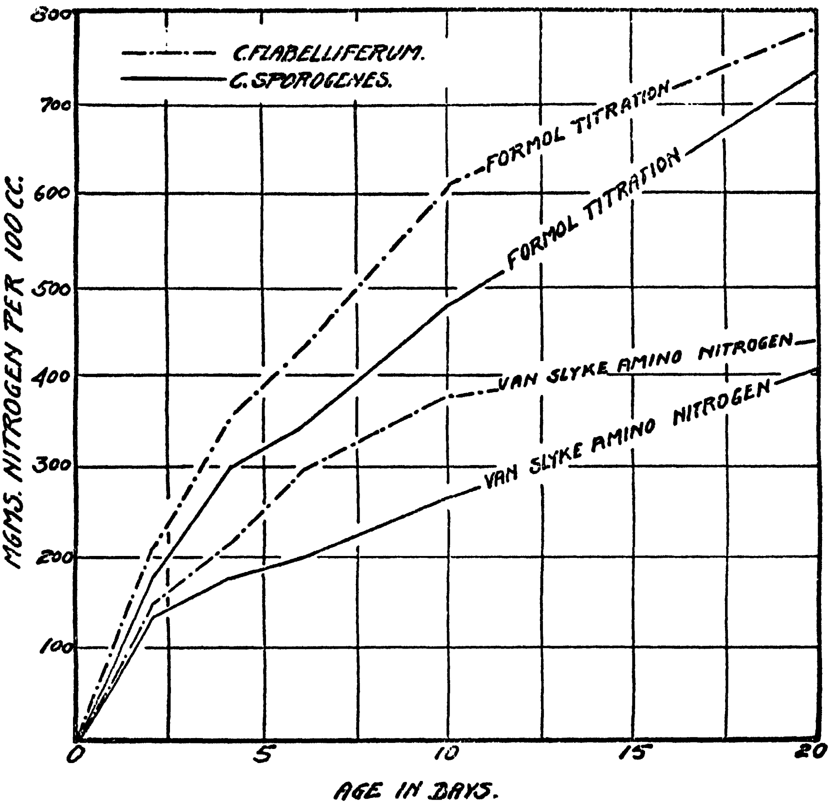

CHART 7. PROTEOLYTIC CHANGES PRODUCED IN GEIATIN AVERAGING EIGHT STRAINS OF C. FLABELLIFERUM AND NINE STRAINS OF C. SPOROGENES

Total nitrogen	1650 mgm. per 100 cc.
Formol titration (initial)	60 mgm. per 100 cc.
Van Slyke amino nitrogen (initial)	63 mgm. per 100 cc.

Therefore, from the data average values of all the strains of each of the two organisms studied have been computed. These results are presented in charts 7 and 8, which show that at any

[2] *C. sporogenes* zone is represented by horizontal lining, *C. flabelliferum* by vertical lining while the zone in common is shown by cross lining.

age the average of the determinations of the change in any property is the greater in the case of *C. flabelliferum.* This

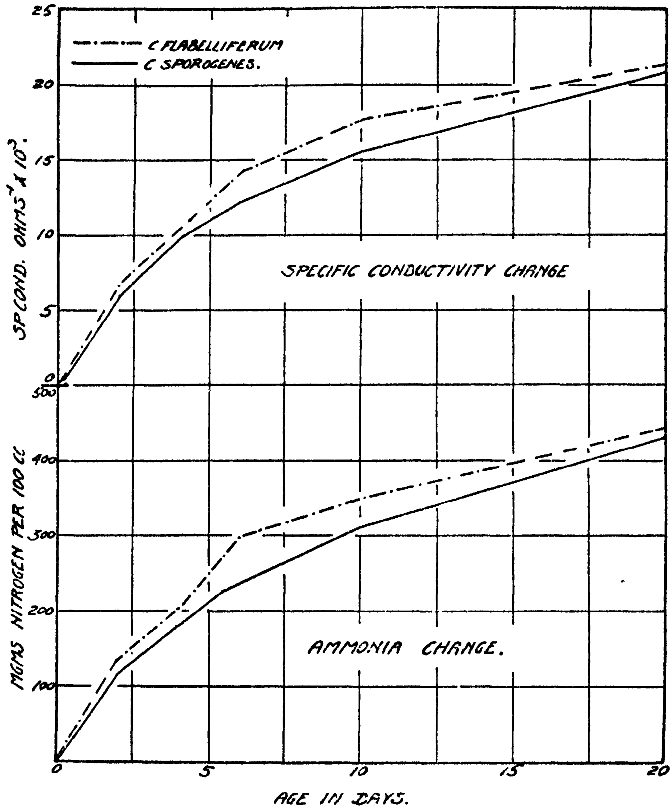

CHART 8. PROTEOLYTIC CHANGES PRODUCED IN GELATIN AVERAGING EIGHT STRAINS OF C. FLABELLIFERUM AND NINE STRAINS OF C. SPROGENES

Total nitrogen..................................	1650 mgm. per 100 cc.
Ammonia, nitrogen (initial)......................	10 mgm. per 100 cc.
Specific conductivity (initial)................	3 5 $\times$ 10^{-3} ohms.$^{-1}$

demonstrates that the distribution of the various strains throughout the plotted zones in charts 4, 5 and 6 is fairly uniform.

DISCUSSION

The results obtained in the preliminary studies carried on with two strains of each anaerobe would point to the conclusion that

C. sporogenes is the more actively proteolytic organism. However, results with a larger number of strains have demonstrated the opposite to be the case. The practice of attempting to make differentiations on the basis of quantitative differences is certainly to be deprecated unless it has been definitely shown that such differences are typical of a relatively large number of strains.

For these particular organisms it appears to be immaterial which property is used to give a measure of the relative amount of proteolysis, since the charts show that in all cases the measurements of changes in conductivity, ammonia, formol titration, and amino nitrogen follow the same general course. The writers, of course recognize that while the above statement is true for these two organisms it may not hold for all proteolytic cultures.

Referring to charts 1, 2 and 3, it further appears immaterial which of these three media be employed in making a comparison of the proteolysis of these two organisms. The close agreement of the sporogenes curves with the flabelliferum curves for conductivity, for formol titration, for ammonia, and for amino nitrogen in any particular medium indicates that the nature of the proteolytic changes produced by the two organisms is essentially the same. The fact that in the three media the sporogenes average curves always slightly exceed the flabelliferum average curves is a striking confirmation of the foregoing conclusion and evidences the consistent behavior of the cultures.

Although differences between two given strains of the two organisms might easily be misinterpreted, the extensive data presented do not suggest any significant differences in the proteolytic activities of *C. flabelliferum* and *C. sporogenes.*

SUMMARY

1. Seven additional isolations of *C. flabelliferum* are reported which conform in distinguishing characteristics with the original.

2. An extensive comparison of proteolytic changes of the new organism with those of *C. sporogenes* is reported.

3. The nature of the proteolytic changes produced by the two organisms appears to be quite similar.

4. The critical survey of the data shows that differences in proteolytic activity between individual strains of the same culture may be more marked than differences between strains of *C. sporogenes* and *C. flabelliferum*. No definite quantitative differentiation between the two organisms is possible on the basis of this study.

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STUDIES IN MICROBIC HEREDITY

V. THE BIOGENETIC LAW OF HAECKEL AND THE ORIGIN OF HETEROGENEITY WITHIN PURE LINES OF BACTERIA[1]

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From the Department of Laboratories, Highland Hospital, Rochester, New York

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Many years ago the biologists, Haeckel and von Baer, made a most interesting generalization which stated that the life history of the individual of a species recapitulated the life history of the race—or in another form, that ontogeny recapitulates phylogeny. This law is still regarded by biologists as valid in a large way, although it cannot be successfully applied in detail, for the simple reason that entire phyletic stages may be suppressed or new non-phyletic adaptive stages interpolated, in a manner quite misleading to one who would unravel the phyletic or racial history of a species from its individual ontogeny.

The general method proposed by these investigators has been particularly fruitful, however, in our studies on the genetic relationships of bacteria. This method consisted, in brief, of making observations over a course of years on selected species (?) isolated from a single cell. For this purpose cultures to be suitable must exhibit a life history expanded into several clean-cut morphologic phases capable of ready reproduction. In other words there must be a clear delineation of the pleomorphic cycle. Figure 1 and diagram 1 show that our K A. diphtheroid strain answered fully this qualification. (See bottom of page 204 for explanation of plate 1.)

Figure 1 at *a*, for example, shows the "normal" or better the dominant phase, in the ontogeny of this culture. This is a rather slender, non-granular, Gram-negative bacillus. At *b* we

[1]Studies III and IV of this series appeared in the Journal of Immunology, Vol. XI, No. 2.

have a very coarse fungoid branching phase which gives rise to the gonidial and zygospore phases, *c* and *C*, and which is Gram-amphophilic. At *d* is a rudimentary branching phase not so coarse as *b*, and at *e* (diagram 1) is another bacillary phase which differs from *a* in being more retentive for the Gram stain. These represent the principal phases in the ontogeny of a pure line culture of this strain. To many bacteriologists it is still known only as the pleomorphic cycle and as such is without general biologic or medical significance.

Haeckel's Law suggests the possibility that these ontogenetic phases may stabilize under certain conditions as stages, variants or races—if one pleases—of this single organism. So stabilized they would then comprise its phylogeny. Indeed this result has been realized experimentally through the mechanism of the reorganization occurring in the sexual cycle. The first results with this strain were published several years ago (Mellon, 1920). The further experimental development of its phylogenetic history is the chief purpose of the present paper.

Previous work with the K. A. strain indicated clearly that under the conditions of the experiment it was possible to develop

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PLATE I

ONTOGENY

Fig. 1. K. A. 95.

DIAGRAM 1. The small circle at the left hand side of the diagram indicates that this strain may be monophasic, i.e., it may multiply apparently to exclusion in the bacillary or so-called normal phase—*a* (Figure 4). The progressive widening of the circles to the right to include phases *e*, *d*, *b* and *c*, indicates that the culture may be bi-, tri- or tetraphasic, etc., and that each of these is capable of reverting to the original phase *a*.

PHYLOGENY

FIG. 3. K. A. 257 variant. Stabilized phase *d* of figure 1.
FIG. 4. K. A. 191 variant. Stabilized phase *e* of diagram 1.
FIG. 5. K. A. 105 variant. Stabilized phase *C* of figure 1.
FIG. 6. Fungoid phase *b*. Not stabilized.

Table of magnifications for photographs

× 1200: Figures 1, 2, 4, 6, 7, 10 and 11
× 1500: Figures 5, 20 and 21
× 2500: Figures 8, 9 and 19
× 3000: Figures 12, 13, 14, 15, 16 and 17

FIG I

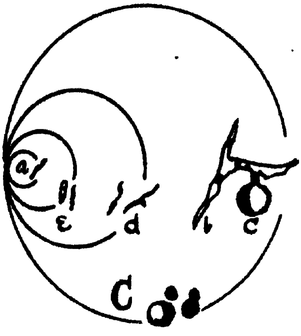

DIAGRAM I

FIG. 3

FIG. 4

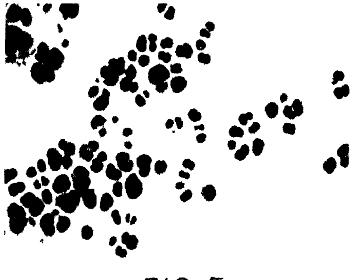

FIG. 5

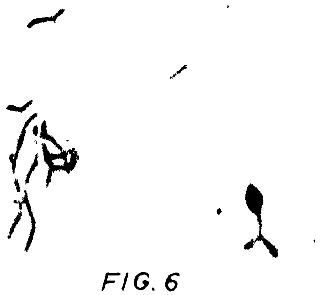

FIG. 6

(Mellon: Studies in microbic heredity)

large numbers of what we spoke of as "giant coccoids," and which we now regard as probable zygospores (1920). The original culture of this pleomorphic strain (fig. 1) will be known as K. A. 95. It was further shown to be possible for these large forms to undergo a double segmentation and yield a true coccus mutant, which will be known in this study as K. A. 105 (fig. 5, plate I). It is now desired to show that the same or similar forms (fig. 2, plate II) will, under other conditions, give rise to a bacillary type of mutant (K. A. 257) (fig. 3, plate I); although the latter is closely related serologically to the antecedent bacillus, their cultural and morphologic characters are so different that one is surprised that this is the case.

EXPERIMENT I: THE MECHANISM OF MUTATION YIELDING STRAIN K. A. 257

The strain in question was isolated in single cell culture by Dr. M. A. Barber in 1919 and was planted in ordinary glycerol broth 0.5 per cent, having a pH of 6.8. As grown at 37° it produced many very large, rather poorly staining giant coccoid forms, originating from the coarse branching phase of the culture (fig. I, *b* and *C*). It was placed in the ice chest for about nine months which caused the broth to evaporate more than one-half. Owing to the lack of viability of other transplants of this strain, this culture was resorted to in an effort to rejuvenate it. At this time it had always grown on Loeffler's at 37° but transplantation under these conditions failed. Placed at room temperature for a week a luxuriant growth of a new type of bacillus was obtained on the Loeffler's slant. Microscopic examination of the old broth cultures showed that apparently no bacilli remained and that it consisted entirely of very deeply staining coccoid forms which varied greatly in size (fig. 2, plate II). The lack of the usual intracellular staining differentiations in these forms may possibly be correlated with the new type of bacillus evolved from them—a very different race from the coccus mutant referred to above.

The large numbers of colonies which germinated on the slant at room temperature left little doubt that the resulting bacillus germinated from these coccoid forms, particularly since none of

the original bacilli appeared. The origin of a new race from these coccoids was definitely proven for another strain of the diphtheria group (1922). The K. A. bacillus, as we originally obtained it, grew sparsely and was Gram-negative (phase *a*), while the large coccoid forms (phase *C*—zygospores) retained the Gram stain decidedly longer and could almost be called Gram-positive, while the variant bacillus which grew at room temperature was also very definitely Gram-positive as was the coccus mutant mentioned above.

Culturally the mutant bacillus, K. A. 257 (fig. 3) grew much more luxuriantly than the original strain, K. A. 95, and developed a buff colored pigment, while the original strain some years later

TABLE 1

	K. A. 257	K. A. 105	K. A. 95	K. A. 191
	pH	*pH*	*pH*	*pH*
Glucose	4.30	5.4	5.3	5.3
Mannitol	7.4	5.7	7.2	7.1
Levulose	4.29	5.6	5.2	5.3
Salicin	5.67	5.6	6.8	6.8
Dextrin	7.4	5.6	6.9	6.8
Inulin	7.4	6.6	7.0	7.0
Sucrose	4.44	5.6	5.7	5.6
Maltose	4.47	5.7	5.7	5.9
Lactose	7.6	6.4	6.9	6.9
Glycerol	7.6	6.8	7.0	7.0

developed a yellowish pigment. Furthermore, the variant strain was very different morphologically in that it showed a marked tendency to rudimentary branching forms, was quite granular, and culturally had a rather wrinkled, more cohesive character than the original bacillus, which was moist and shiny. These cultural differences were furthermore associated with the acquisition of the fermentation of salicin, as is seen in table 1, which gives the sugar reactions of the various strains.

EXPERIMENT II: THE ORIGIN OF STRAIN K. A. 191

Strain K. A. 191 (fig. 4) was derived from the pleomorphic K. A. 95 as follows: The pure line K. A. 95 had been kept on a blood

agar slant for some months in the ice chest. The growth was luxuriant but the pleomorphism slight, as was the rule on this medium. The rather fitful viability of this strain has already been mentioned, and it was notoriously weak on blood agar in contrast to a prolonged viability on Loeffler's medium where the gonidial or giant coccoid forms were usually numerous.

Most of the attempts to revive the blood agar strains failed whether transfers were made at 37° or 20° and regardless of the media tried; but for some unknown reason one tube which had failed to grow at 37° did finally grow at 20° on a blood agar slant. Morphologically the strain was identical, and it was at first thought that the original strain had been recovered; but the fact that it gradually developed a yellow pigment, even in young cultures, that it also grew luxuriantly on Loeffler's medium where its pleomorphism was greatly diminished, that it failed to develop numerous gonidia (giant coccoids) in serum broth, and that it was definitely—if not strongly—Gram-positive, made it clear that we were dealing with a variant.

Serologically it proved to be mutually reciprocal with K. A. 95, but the fact that it failed to form the large gonidia (zygospores) in appreciable numbers made it unsuitable for the coccus mutation experiments as originally described for it. Here is a fact of the *greatest importance in mutation experiments* because it may well account for the failure of one observer to repeat the work of another when he *fancies* he is working with the identical strain, merely because it is serologically identical.

When it is better appreciated that mutation is not apt to take place except through the medium of a special developmental stage of the culture, attention will be directed to developing an environment that will evoke such a stage. If this cannot be done, the culture is apt to remain very stable. Strain K. A. 191 is one of the few variants in which I could not be sure of the mechanism because the pleomorphic forms were not present in sufficient numbers to trace it readily.

Phylogenetically it is apparent that K. A. 191 (fig. 4) represents a stabilization of stage *e*) of the ontogenetic cycle (fig. 1 and diagram 1); K. A. 257 (fig. 3) represents stage *d*; K. A. 105 (fig.

5) represents stage *C*. The coarse fungoid stage *b* (fig. 6) has never been stabilized with this species, although as reported a number of years ago a true streptothrix stage of a fuso-spirillary complex was stabilized (Mellon, 1919a).

EXPERIMENT III: THE EFFECT OF SELECTIVE ENVIRONMENT IN THE DEVELOPMENT OF THE PLEOMORPHIC PHASES OF THE K. A. STRAIN

Of a wholly different order from this *permanent* stabilization of morphologic type, is the *temporary* reproduction that can be induced in these ontogenetic (pleomorphic) phases by a selective environment. Yet the latter is of importance if for no other reason than as conclusive proof that these pleomorphic forms are capable of multiplication. In such event they cannot properly be viewed as involution forms.

For example the rudimentary branching stage *d* in the original culture will reproduce *as such* to a limited extent if the pleomorphic mixture is transplanted to tall tubes of glucose ascitic broth where the conditions are largely anaerobic. Stage *a*, which is the normal bacillary stage, will not reproduce noticeably under these conditions. Transfer of these forms to the original conditions brings a return of the original picture. *No stabilization has occurred.*

We have frequently observed, too, the temporary reproduction of phases *b* and *C* under the following conditions. These phases developed readily on Loeffler's slants at 37° and seemed directly connected with the prolonged viability of the organisms, since old cultures on media which did not yield phase *C* were usually non-viable.

Even in old cultures on the Loeffler's medium the bacillary phase *a* was often non-viable, and on transplant to fresh media phases *b* and *C* often grew temporarily to the practical exclusion of the bacillary phase, as seen in figures 7 and 8. However, after twenty-four hours phase *b* began to dissociate the normal bacillary phase *a*, which thereafter completely dominated the picture. This reproduction could be observed directly under the

warm stage much after the manner of germination observed by Gardner (1925) with the "Y" forms of the dysentery group.

It should be perfectly clear then that any one ontogenetic phase of a culture may multiply almost to the exclusion of the other phases under conditions suitable for it, without mutation having occurred; but it should be equally clear if the multiplication is to be continued under a variety of conditions that the reorganizations incident to the sexual cycle must condition it, as indicated in the genetic origin of the mutant strains, K. A. 105 and K. A. 257.

Inasmuch as the K. A. strain is a diphtheroid it is of great interest to summarize briefly the work of others with the well known Park No. 8 diphtheria strain, since their collective observations point definitely to the existence of an ontogenetic cycle for this strain. Many years ago Dr. Anna Williams (1910) threw suspicion on the involutionary nature of the "branching involution forms," so-called, of this strain when she showed by direct observation that their metachromatic granules seemed to fuse before fission took place, and this fission she interpreted as a primitive sexual process. Moreover these branching forms were the only ones in the culture to show active growth and division under the condition of the experiment.

Just recently Martin, Loiseau and Gidon (1924) have noted that the same strain under partially anaerobic conditions yielded long filaments with lateral branches. Although this development begins after twenty-four hours and reaches its maximum by the eleventh or thirteenth day, the forms are still strongly Gram-positive, even after sixty days at 37°. It is significant that they consider these forms *not as evidence of degeneration*, but as normal developments under partially anaerobic conditions.

Again Heinemann (1917) working with this same strain noted that its almost complete loss of toxin producing power was correlated with a radically changed morphology. He found it to be reproducing in streptococcus and diplococcus form which bred true on glucose agar. Transplanted to Loeffler's medium the bacillary form of normal virulence was restored. It is obvious that this Park No. 8 strain undergoes important biologic alterations which are associated with its ontogenetic or pleomorphic

cycle and as such is strictly comparable to our K. A. strain, further evidence of whose heterogeneity will now be considered.

THE ORIGIN OF HETEROGENEITY IN BACTERIAL CULTURES

These experiments indicate clearly that the pleomorphic forms of a bacterial culture are *in a sense* independent entities capable of multiplication *as such* for a limited number of generations, at least. If—as will be shown—these phases have other physiological differences which emphasize their individuality, then we are forced to the conclusion that such cultures are in reality heterogeneous assemblages of individuals whose origin is the pleomorphic cycle.

In addition to the fact that these individual phases may reproduce as such, it is important to note that the mechanism of reproduction is not always the same. Phases *a* and *e* reproduce by transverse fission; phase *b* by branching, gonidial and zygospore formation (fig. 1, 6 and 7); and phase *C* by budding (figs. 8 and 9 at *a*). The latter being an ontogenetic phase, it proved impossible of course to isolate it, yet multiplication by budding could be observed directly under the microscope. Moreover the inoculation material contained relatively few of these circular forms (cf. fig. 1); yet in the transplant they dominated the morphologic picture (fig. 8) until they were overgrown later by the normal bacillary phase. The latter originated from the coarse branching phase *b* of figure 7. Here is definite proof of *reproductive heterogeneity* in a pure line culture.

The increase viability of phases *b* and *c* indicate cultural heterogeneity in respect to this character. As indicated, the origin of phase *a* from *b* and *c* prevented the cultures from dying out altogether. Old cultures of the original strain which did not contain these phases stained poorly, if at all. This was the case when the strain was grown on plain or blood agar instead of Loeffler's; but on the latter medium where the cultures were viable for many months, or even for more than a year, phases *b* or *c* were the only staining forms in the culture. Variant strain K. A. 191, representing phase *e*, maintains viability with little or no reproduction of the special phases on ordinary media or Loeffler's;

FIG. 2

FIG. 7

FIG. 8

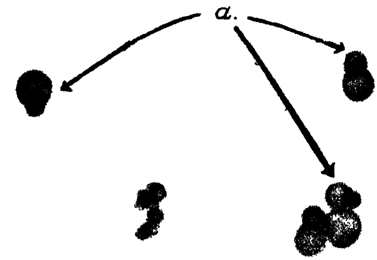

FIG. 9

FIG. 10

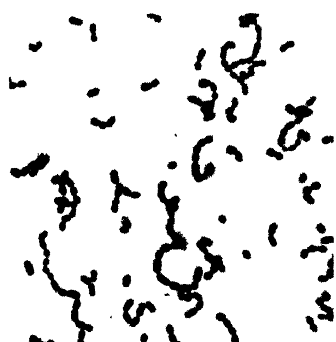

FIG. 11

(Mellon Studies in microbic heredity)

yet on egg media these organisms frequently appear in fair numbers, showing that the capatiy for their formation had been suppressed but not entirely lost.

The variant strain K. A. 191 had acquired pigment formation and grew readily at 20°, which was not the case with the original strain. This temperature adaptation was suggestively associated with the gonidial phase *c*, since when these forms were present in an old culture it germinated readily at 37° but not at all at 20°, while old cultures which contained only the bacillary phase *a*, germinated readily at 20° but failed to do so at 37°. Such a fact might be of considerable importance for the initiation of infection with these organisms.

It will be recalled that the different phases of the ontogenetic or pleomorphic cycle reacted somewhat differently to the Gram stain. The coccoid and fungoid phases retained the Gram stain better than did the simple non-granular bacillary phase. It is not surprising then that the phylogenetic races should exhibit a corresponding variability.

The Gram amphophilism of the individual zygospores (phase *C*) is strikingly repeated in their lineal descendant, the coccus mutant strain K. A. 105 (fig. 5). Although today it would be called a frank Gram-positive coccus it is never quite free from the Gram-negative forms, which greatly increase in number when the culture is two or three days old.

They are small and appear to arise by budding from the larger Gram-positive forms. Even in younger cultures one-half of the coccus is frequently Gram-positive and the other half Gram-negative. The ontogeny of this coccus strain reveals under suitable conditions a succession of phases beginning with large coccoid forms, tetrads, and even at times bacillary forms (fig. 5). In short the cycle is reversed from that occurring with the pure line *bacillary* culture, but the interpretation is precisely the same. This strain produces a viridans type of hemolysis and many strains of *S. viridans* are as much diphtheroid in their morphology as coccus. That this interpretation holds just as nicely for the ontogeny of the cocci as for the bacilli we have experimentally shown in Study III of this series (Mellon, 1925c).

Figure 10 shows the morphology of single cell culture of an organism in a twenty-four-hour culture on a blood agar slant, which grows in ascitic fluid or broth as figure 11. One is a diphtheroid, the other a long chained streptococcus. The literature contains numerous instances of strains of this sort of which I shall quote but one, that of Ohlmacher (1902) who showed that streptococci and *B. coli* may germinate as practically pure rods or cocci, depending on the environment. The reversibility of the phenomenon caused a negative biologic evaluation to be assigned it by bacteriologists.

Its real significance we have experimentally demonstrated, first with the J strain (1917) and later with pure lines of the K. A. strain (1920). The actual mutation from diphtheroid to streptococcus that has been accomplished in these instances is a realization of the *potential phylogeny* represented by the ontogenetic phases of the culture. *It means, in short, that stabilization of these phases is a possibility, but only through a definite mechanism such as we have described.*

Again, the phenomenon of one phase of a culture producing acid and another phase alkali has already been set forth in Study II of this series (Mellon, 1925b). It represents one more aspect of heterogeneity that seems explained on these grounds. There is evidence, too, that alterations in virulence may often represent an aspect of a heterogeneity fundamentally no different from the others. In Study III of this series (Mellon 1925c) we have observed sudden acquisition of virulence in an alkaligenes strain that was definitely associated with a phylogenetic dissociation. Heinemann (1917) several years ago made a similar observation with the Klebs-Loeffler bacillus. This strain showed strikingly the reversible coccus and bacillary stages which could be correlated with differences in virulence. Latterly Amoss (1925) and Reimann (1925) show that pneumococci may be heterogeneous with respect to virulence, although they give no indication of the origin of that heterogeneity. It seems possible that this conception might have yielded much to the field of practical epidemiology had it not been restrained by the preconceptions to which I have referred.

FIG. 12

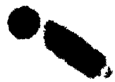

FIG. 13

FIG. 14

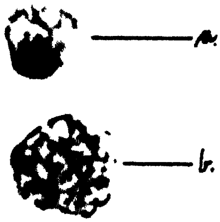

FIG. 15

FIG. 16

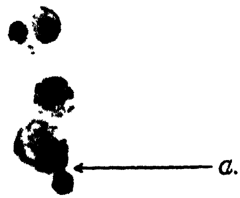

FIG. 17

FIG. 19

FIG. 20

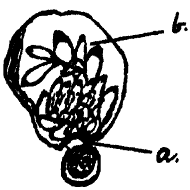

FIG. 18

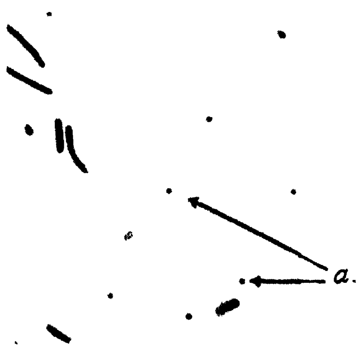

FIG. 21

(Mellon: Studies in microbic heredity)

ATYPICAL REPRODUCTION OF B. COLI

Inasmuch as this paper has dealt with the different methods of reproduction that may occur with different phases of a single culture, it seems desirable to include here a few observations on the capacity shown by *B. coli* to bud under suitable conditions. These conditions are described in detail on page 487 of Study I of this series (1925a) and the strain under consideration is the Nx one of that paper. The forms, in addition to being larger than normal as a result of the conditions under which they were grown, were stained intravitally which avoids fixation artifacts. Moreover they were magnified from 1500 to 3000 diameters, all of which accounts for their huge size.

In figure 12 we have a bipolar organism, and in figure 13 a unipolar one with early constriction of a terminal gonidial granule. In figure 13 no division wall is present as in strict fission, while in figure 14 the snaring off of a primary bud is followed by a similar process with a secondary bud. In figure 15 are two microgonidial forms which originated from the filaments that were common with this strain. Note typical early budding at *a*, with the process more advanced in figure 16 where constriction is beginning. In figure 17 at *a* the bud is about snared off and it is particularly noteworthy that the chromatin of the maternal cell participating in the division is thrown into a skein where twenty loops can be counted in the negative. It is almost a karyokinetic evolution of the nuclear matter. Diagrammatic representation of the same is seen in figure 18 where a secondary loop formation of the chromatin is seen at *b*. Note crater formation at *a*, typical for the germinating forms of this species. In figure 19 the bud is practically freed as the daughter cell containing the deeply staining chromatin.

The method of reproduction shown in the large forms of *B. coli* (figs. 12 to 14, inclusive) can be seen in other strains of coli where the bacilli are of normal size as they grow in ordinary broth. Figures 20 and 21 at *a* show this type of reproduction which represents an intermediate stage between true fission and budding. The resulting gonidia are seen at *a* in figure 21. Although the

latter usually elongate to bacilli they may occasionally continue to grow as a diplococcoid race of *B. coli.*

This is a confirmation of the work of Hort (1917) who observed directly under the microscope similar reproductions with *B. typhosus.* Again we see that the characterization of bacteria as fission fungi must be regarded as only relatively true.

DISCUSSION

The half knowledge of these essential points bequeathed us by tradition has largely contributed to the confused and dogmatic position taken by certain sections of orthodox bacteriology in regard to them. The doctrines of monomorphism have developed no intelligent conception of the manner in which new types (developmental stages) might be *potentially present* in perfectly pure line cultures. In the past, experiments that have realized this potentiality in the dissociation of new types out of the pleomorphic cycle, have usually been prejudged on the ground that pleomorphism among the bacteria had no meaning, and the new type must have persisted *as such* in the original culture.

In recent years practical considerations in respect to the agglutination reaction leave little doubt that many bacterial cultures are heterogenous; yet so great has been the obeisance to the *involution* anachronism that it is not yet realized that the real key to the origin of this heterogeneity is inextricably associated with the "involution" forms themselves. In short, heterogeneity is the physiological counterpart of bacterial pleomorphism, and as such is a proof that the latter has important biologic connotations.

The question arises whether we shall consider as mutants the new types that may at times arise in association with sexual reorganization. To Enderlein (1925) the physiologic and cultural changes that he has shown to result from sexual reorganization in *B. cholera* constitute a new "growth generation" and cannot be considered a mutation. On theoretical grounds he inclines to the view that most of our bacterial mutants, so-called, fall in the same class. It is my opinion that these rejuvenation changes need not preclude the occurrence at times of more far reaching

ones in the same anlagen, i.e., in the gonidia or zygospore. It appears to be largely a matter of the extent of the reorganization which their environment permits to occur in them. It would be difficult to assert that the reorganization resulting in slight changes is *fundamentally* different from that producing more far reaching changes of character.

Certainly when one is able to derive organisms as different culturally, morphologically and serologically as are the strains under consideration, it would be difficult to know what to consider mutants if these are not so regarded. With no disposition to take a dogmatic view of the situation I must tentatively regard the *stabilized* stages in the phylogeny of the organisms as variants or mutants if the new characters are *constant.* If the organism gradually reverts as it ages I would then regard the "new" organism as a "growth generation."

FERMENTATION REACTIONS

The cultures were incubated for from seven to ten days at 37°C. in sugar free broth to which 1 per cent of the various sugars and bromothymol blue were added to a slight greenish tint. Good growth was obtained in all tubes. The pH of the uninoculated controls without any sugar was 7.0; of the inoculated controls 7.0 to 7.02. The readings were colorimetric except in case of doubt when they were checked with the hydrogen electrode. They were made after seven days at 37°C. and again after seven days more at 20°C.

The tubes which produced acid in from seven to ten days usually produced alkali after that period if they had not already shown it. Salicin was somewhat of an exception with the K. A. 257, inasmuch as the formation of acid was rather slow, but in fourteen days it was very definite, both by titration and colorimetrically. It will be seen that this was the only qualitative fermentation difference between K. A. 95, K. A. 191 and K. A. 257. With K. A. 105, however, mannitol, dextrin, lactose and probably insulin were fermented, which is not the case for the other three strains.

SEROLOGIC REACTIONS

Immune sera were developed in rabbits by intravenous inoculation of killed cultures of the various strains which had been isolated from a single cell, and grown on plain agar. From four to six inoculations were given over a course of two weeks. Sera were tested from seven to ten days later and if of sufficiently high titre the animals were bled and the sera put in the ice chest. Before inoculation the rabbit's sera were tested with all strains, which served as normal control sera. These were repeated when the experiments were done. The tubes were incubated two hours at 37° and read, then put in the ice chest over night and read a second time. After twenty-four hours more at 20° they were again examined.

The adsorption technique was the same as detailed in Study III of this series. K. A. 95 antiserum was developed at a time when the original bacillus was not pigmented and did not grow very luxuriantly. K. A. 191 antiserum, was made a couple of years later when the organism had acquired pigment formation, grew more luxuriantly and had lost to a large extent its capacity to form coccoids.

In the table, C indicates that agglutination is complete; 3+, 2+ and 1+ indicate a progressively lessened amount of it; – is negative; a blank space indicates no test.

Analysis of tables 2 and 3

From tables 2 and 3 it is clear that K. A. 191 and K. A. 257 agglutinate to full titre in each other's sera. Since K. A. 191 agglutinates to full titre in K. A. 95 serum it is probable that the reverse reaction would have been similar had it been possible to carry it out. On the other hand there is no evidence of cross agglutination between the coccus K. A. 105 and any of the bacillary strains, using the *usual technique.*

However, in Studies III and IV of this series (Mellon, 1925a and 1926) we have shown that it is possible at times to detect the presence of small amounts of group agglutinins by employing a temperature of 20° instead of 37° for incubation. Accordingly

TABLE 2

Agglutination reactions

ANTIGEN	TITRE OF THE HOMOLOGOUS SERA									NORMAL SERUM			NaCl 0.85	ANTISERUM
	1–20	40	80	160	320	640	1280	2500	5009	1–20	40	80		
K. A. 95	C	C	C	C	C	3+	–	–	–	–	–	–	–	K. A. 95
K. A. 257	C	C	C	C	C	C	C	3+	–	–	–	–	–	K. A. 257
K. A. 191	C	C	C	C	C	C	3+	–	–	–	–	–	–	K. A. 191
K. A. 105	C	C	C	C	3+	–	–	–	–	–	–	–	–	K. A. 105

TABLE 3

Cross agglutination reactions

ANTIGEN	1–20	40	80	160	320	649	1280	2500	5000	NORMAL SERUM			NaCl 0 85	ANTISERUM
										1–20	40	80		
K. A. 191	C	C	C	C	2+	–				–	–		–	K. A. 257
K. A. 105	1+	1+	–	–	–	–				–	–	–	–	K. A. 257
K. A. 95*													–	K. A. 257
K. A. 257	C	C	C	C	C	C	C	C	–	–	–	–	–	K. A. 191
K. A. 105	1+	1+	1+	–	–	–	–			–	–	–	–	K. A. 191
K. A. 95*													–	
K. A. 257	±1	±1	–	–	–	–	–			1+	–	–	–	K. A. 105
K. A. 191	±1	±1	±1	–	–	–	–			1+	–	–	–	K. A. 105
K. A. 95	±1	±1	–	–	–	–	–			–	–	–	–	K. A. 105
K. A. 105	–1	–	–	–	–	–	–			–	–	–	–	K. A. 95
K. A. 191	C	C	C	C	C	3+				–	–	–	–	K. A. 95
K. A. 257†													–	K. A. 95

* Not done because K. A. 95 did not regenerate when the variant K. A. 191 developed from it.

† Not done because K. A. 95 serum was exhausted at the time variant K. A. 257 was dissociated.

this was done. When K. A. 105 was run against K. A. 257 serum at 24° no agglutination could be detected, but K. A. 105 vs. K. A. 191 serum showed a definite flocculation in six tubes at the end of one hour.

Although the flocculation could be readily seen with a hand lens at the end of twenty-four hours there was little, if any, increase in it, and actual precipitation was scarcely noticeable. No agglutination occurred with the normal serum of K. A. 191 at 24°. It is desirable to employ a temperature of 20° rather than 24° as the results are usually sharper. At the time the tests were done the room temperature was 24° and it was not convenient to use 20°, especially when the adsorption test was conclusive.

Analysis of table 4

As seen in table 4 the antisera of K. A. 257 and K. A. 191 reciprocally adsorb to exhaustion the homologous agglutinins. K. A. 191 and K. A. 257 do not agglutinate in K. A. 105 serum even with the 20° technique, and the adsorption results are negative. K. A. 105 does not agglutinate in K. A. 257 serum, neither does it adsorb any agglutinins for K. A. 257 from this serum. On the other hand at 24° K. A. 105 does show partial agglutination in K. A. 191 serum and on adsorption reduces the titre of the latter from a 3+ in a dilution of 1280 (table 2) to a ±1 in a dilution of 640.

This result is of striking interest, since it appears that a pigmented diphtheroid bacillus not affecting blood agar produces group agglutinins for a frank diplococcus, non-pigmented, but producing a viridans type of hemolysis on blood agar. The sugar reactions of the two strains are also divergent. Although the reduction in titre of the serum is quantitatively not striking, it is appreciable, definite and constant for this serum.[2] The fact

[2] Several other animals were immunized with K. A.-191. These sera were adsorbed with K. A.-105 antigen and the reactions with the homologous strain varied from demonstrable ones to one that is practically inappreciable. These differences in reaction in different animals where small amounts of group agglutinin are concerned find detailed explanation in Study No. III of this series (loc. cit.).

TABLE 4

The results of adsorption

ANTIGEN	1–20	40	80	160	320	640	1280	2500	NaCl 0.85	SERUM
K. A. 257	–	–	–	–	–	–			–	K. A. 191 c Ant. 257*
K. A. 191	–	–	–	–	–	–			–	K. A. 191 c Ant. 257
K. A. 105	–	–	–	–	–	–			–	K. A. 191 c Ant. 257
K. A. 191	–	–	–	–	–	–			–	K. A. 257 c Ant. 191
K. A. 257	–	–	–	–	–	–			–	K. A. 257 c Ant. 191
K. A. 105	–	–	–	–	–	–			–	K. A. 257 c Ant. 191
K. A. 105	–	–	–	–	–	–			–	K. A. 191 c Ant. 105
K. A. 257	C	C	C	C	C	C	C	3+	–	K. A. 191 c Ant. 105
K. A. 191	C	C	C	C	C	±			–	K. A. 191 c Ant. 105
K. A. 105	–	–	–	–	–	–			–	K. A. 257 c Ant. 105
K. A. 257	C	C	C	C	C	C	C	3+	–	K. A. 257 c Ant. 105
K. A. 191	C	C	C	C	±				–	K. A. 257 c Ant. 105
K. A. 105	C	C	C	C	C	1+	–			K. A. 105 c Ant. 257
K. A. 191	–	–	–	–	–	–	–			K. A. 105 c Ant. 257
K. A. 105	C	C	C	C	C	2+	–			K. A. 105 c Ant. 191
K. A. 191	–	–	–	–	–	–	–			K. A. 105 c Ant. 191

* c stands for "adsorbed with;" Ant. for "antigen."

that there is no suggestion of such a result with strain K. A. 257 (which agglutinates and adsorbs reciprocally with K. A. 191) may mean that it is not precisely correct to base serologic identity on reciprocal adsorptions. It is probably beyond the realm of possibility to say that the antigenic complexes of two unknown strains are identical, since it would be impracticable to test out the antisera of each with a host of other strains.

The experiment is quite revealing in another direction, showing as it does that a failure of two culturally and morphologically diverse organisms to cross agglutinate does not preclude their genetic origin from a common ancestor. It is pertinent to refer to a study made by us several years ago (1919b) in which we showed that the spore and vegetative phases of the same microörganism yielded two distinct agglutinogens, from which were produced antisera that were not reciprocally adsorbed to any appreciable extent.

Strain K. A. 105 was dissociated six years ago; strain K. A. 257 was split off three years ago, and K. A. 191 four years ago. They have been quite constant in every respect since this dissociation. In order further to control this experiment an attempt was made to adsorb K. A. 191 serum with a hemolytic streptococcus (A-12) isolated at autopsy. The result was completely negative, so that we have no evidence that the cross adsorption of K. A. 105 for K. A. 191 serum could be referred to some nonspecific adsorbing factor characteristic of the streptococcus group as a whole.

Grateful acknowledgment is made to Miss Elizabeth Yost for painstaking assistance in the serologic part of this study, and to Dr. A. P. H. Trivelli of the Eastman Kodak Co., for valuable suggestions with certain of the microphotographs.

CONCLUSIONS

1. The biogenetic law of Haeckel is capable of application to the genetic history of bacteria.

2. The various phases of the pleomorphic cycle represent the ontogeny of a strain; when the phases are stabilized through mutation they collectively represent the phylogeny of this strain.

3. The bacterial classifications that *widely separate* the cocci from the bacilli do not find genetic support, irrespective of the practical value of such artificial division.

4. Group and specific agglutinogens have been demonstrated between stable races of bacilli and cocci.

5. Complete serologic reciprocity has been shown for two races of bacilli, morphologically and culturally quite diverse.

6. Considerable biologic diversity has been shown to exist among the various phases of the pleomorphic cycle. On this fact can be appropriately based the heterogeneity of pure line cultures.

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STUDIES IN MICROBIC HEREDITY

VI. THE INFECTIVE AND TAXONOMIC SIGNIFICANCE OF A NEWLY DESCRIBED ASCOSPORE STAGE FOR THE FUNGI OF BLASTOMYCOSIS[1]

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The observations to be reported deal with the evidence of an ascospore stage for the simpler types of the group of fungi that are regarded as etiologic for the lesions of blastomycosis. Other forms of special resistance such as the so-called "dauernzellen" have also been observed. The demonstration of asci for this group has a distinct bearing on their rather uncertain taxonomic position and in addition offers an explanation for the noteworthy chronicity of the disease as it occurs in man. Furthermore, it may help to explain the recrudescences of the disease after apparently yielding to treatment. A preliminary note dealing with this observation has already appeared (Mellon, 1924).

The observations of Ricketts (1901) made him favorable to the possibility of ascospore formation but, with the exception of his third or higher group, he could not convince himself that such occurred. Even with this third group he observed what appeared to be terminal ascospores only occasionally and there was some question of their nature. He apparently has not

[1] From the standpoint of the mycologist the term "blastomycosis" or "blastomycete" may be open to considerable objection on account of its taxonomic ambiguity; yet in order to link up this study with a disease that clinically and pathologically is a distinct entity I shall refer to it in this paper by its usual name so as not to confuse those in medical biology who know it by no other name except, perhaps, oidiomycosis. This latter is not a taxonomic improvement on blastomycosis. If I were to suggest a name on the basis of this study it would be some species of the genus endomyces. Certainly from the mycologic point of view blastomyces is too general a term.

included any such among his photographs. His large forms, which contained very numerous intracellular bodies, do not constitute in his judgment sufficient evidence of endosporulation. The lack of a double contoured membrane for the inclosed bodies, their relative inability to stain, and their great number, are points which fail to correspond with the observations which we have made. The typical morphology of our bodies, combined with the prolonged viability of the cultures in which they are found, leaves no reasonable doubt that they are true ascospores.

Other writers, notably LeCount and Myers (1907), Stober (1914) and Wade (1916), describe very numerous, rather minute forms in the tissues of the host which suggest an endosporulation. They also find an occasional empty shell in the tissue, which LeCount and Myers believe supports the endosporulation theory; but Wade interprets such formations as the result of tissue activity and not as the product of vital activity on the part of the parasite. Furthermore, the small size of the parasite results, in his opinion, from a variation of the gemmation process, which he speaks of as a "frantic multiplication."

Although Wade's view of the origin of the minute forms is more probable, our cultural studies will show that LeCount and Myers' interpretation of the shell-like capsules is deserving of consideration. True ascus formation occurring in our cultures has not yielded many minute forms, but only structures containing four normal sized cells.

Furthermore, the direct observations of Hamburger (1907) show that although minute forms originate by rupture of a large cell they are not viable, and, properly, as I believe, he does not regard them as the result of ascus formation. Whether it is possible for minute germination units to be formed by a type of endosporulation not now recognized among these fungi is of course a question that only the future may decide.

It is to be recalled that Ophüls (1905) has shown that the parasites of coccidoidal granuloma—the blastomycotic disease occurring on the Pacific Coast—reproduce rather characteristically by endosporulation in the tissues. This feature, constituting one of the chief differences from the eastern cases, may

come to be regarded as one of degree only, in light of the observations to be here reported.

SOURCE OF CULTURES

The organisms studied corresponded to Types I and II of Ricketts. Type I proliferated chiefly by budding, although mycelia sometimes developed as the cultures aged. In Type II the mycelial element initiated the growth of the organism by fastening itself to the media and from this a profusion of buds quickly developed. Mycelial arborizations frequently penetrated the medium. Both these cultures were isolated from the lesions of the same patient.

Type I was easily recovered in purity from the young lesions (fig. 1, at *a*), while Type II came invariably from the older lesions where its isolation was effected with much difficulty. This double isolation may be of significance, suggesting that the conflict between host and parasite evolved a different type in the older lesions. The possibilities of this important genetic consideration has been made the subject of experiments whose results will be reported in a separate communication.

Figure 1 shows the lesions about the face of the case from which the organisms were isolated. Similar lesions were found on the arms and legs. From the young lesions at *a* a Type I yeast was obtained. The lesion was closed and was surgically removed *in toto* under aseptic conditions. From the border of an older lesion on the leg Type II was isolated, but Type I did not grow; yet from a young lesion not over a month old and removed only a few inches from the older one, Type I was readily isolated. Clinically and histologically these lesions were typical of the disease. The recognition of the asci in the cultures was in essence a by-product of an inquiry into the biologic nature of secondary colonies among the bacteria.

It was desired to study at first hand these secondary growths among fungi, where they are common, with the idea of correlating their significance if possible with similar developments among the bacteria. It is rather generally recognized that these secondary colonies among the fungi represent a distinct

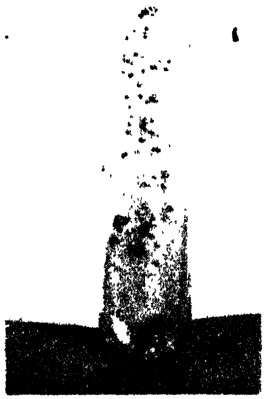
phase in the life history of the fungus, and one of our previous studies on variability (Mellon, 1922) made it appear that they have here the same significance.

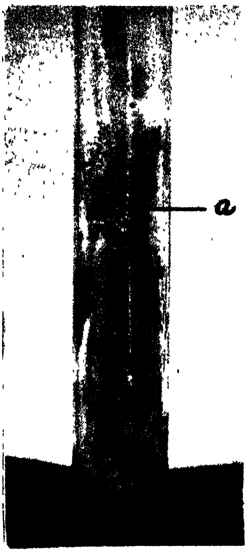
It was in these secondary colonies exclusively that the forms of special resistance such as ascospores, oidial forms, chlamydospores and "dauernzellen" were found, and the fact that their development was often a matter of weeks or even months may explain why asci have not been observed before. Most of our studies were conducted on Type II where the secondary colonies showed to special advantage and were readily recognized as pigmented elevations springing from a background represented by the primary growth. Their pigment was usually black or very dark brown but in some instances the secondary colonies were buff, or even pink in color.

Figure 2 shows to good advantage the black colonies on a background composed of the relatively colorless primary growth. Figure 3 shows a chagreened secondary type of growth with the black secondary development at *a*. Figure 4 shows at the bottom of the tube the penetration into the media's depths of beautiful dendritic arborizations, taking origin apparently from the same structures as the black secondary colonies on the surface. These are the intermediate or Type II fungi. Figure 5 shows the secondary developments of Type I. The ulcerated or pock marked looking areas are tough in consistency, and have a pleomorphism, microscopically, which identifies them as developmental phases in the life cycle of the organism.

Figure 6 is a Type II organism showing clusters of colorless and pigmented secondary colonies. These colonies were usually very tough and could be removed *en masse* from the media. Microscopically they are usually dominated by the oidial form, so-called, often pigmented, which occurs singly or in threads or pairs. They are usually much larger and differ in shape from the normal forms of the organism. They have also a much thicker capsule and stain with great intensity.

These morphologic differences are clearly seen in figure 7 where the small oval organism at *a* represents the so-called "normal" form of the parasite. The oidial form may reproduce





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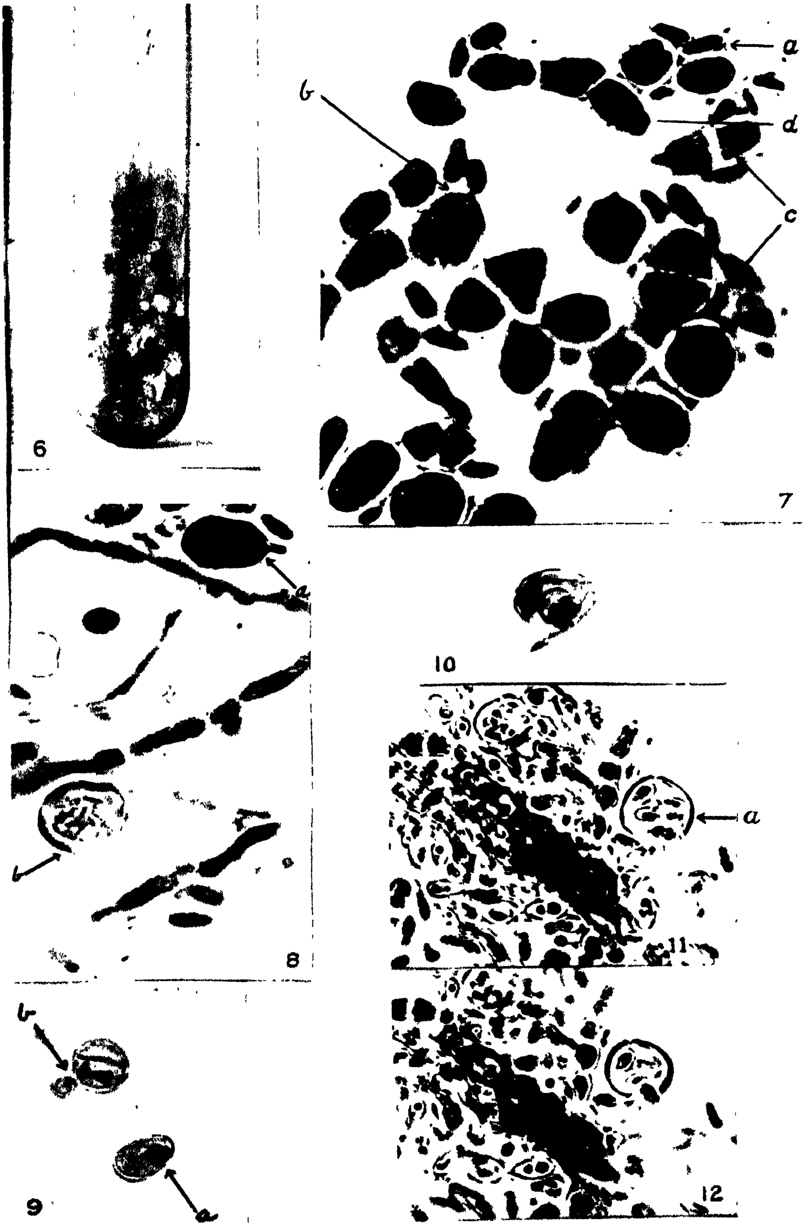
(Mellon: Studies in Microbic Heredity)



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by budding as at *b* although the forms at *c* suggest a transverse fission. Subsequent divisions may form a thread, the beginning of which is seen at *d* in figure 7 and also in figure 21. Although the transverse division wall between these cells suggests pure fission, the cell appears primarily to put out a bud, which is then separated from the maternal cell by a transverse division wall. Although finality on this point awaits further direct study of the germinating forms, we have seen no evidence that these "oidia" arise by fragmentation of a mycelial filament as some authors believe.

In figure 8 the mycelial phase is observed in addition to an oidial cell at *a* showing early budding, and an encapsulated form at *b* whose possible significance will be considered later. In figure 9 (Type I) we have what corresponds to the "dauernzellen" of H. Will (1895). In figure 9 the nucleus-like structure seen at *a* is apparently capable of undergoing typical gemmation as seen at *b*. Note the transverse rent in the capsule. When the large intensely staining oidial forms germinate they may put out from one to four or five of these buds.

In figure 10 one sees a doubly contoured form of the organism within a large form, a rent in whose capsule is indicated by the triangular refraction lines inclosing it, and by the stripping of the capsule itself. This form is structurally different from the four-celled ascus characteristic for this species. It is probable that this preformed cell is destined to bud forth from the maternal cell as an oidial stage, further stages in which process are detailed in connection with figures 18, 20, 21 and 23.

The secondary colonies which are red or buff color usually consist of these rather large forms having a transverse rent in their capsule as if something had escaped. The edges are frequently folded back. In contradistinction to the black secondary colonies the red colonies may not be viable. If they contain well stained forms, however, they are always viable.

The black colonies are noted for their great viability—in fact if it were not for their development we should have long since lost this culture which has now been under observation for five years. Under conditions where only the normal forms develop,

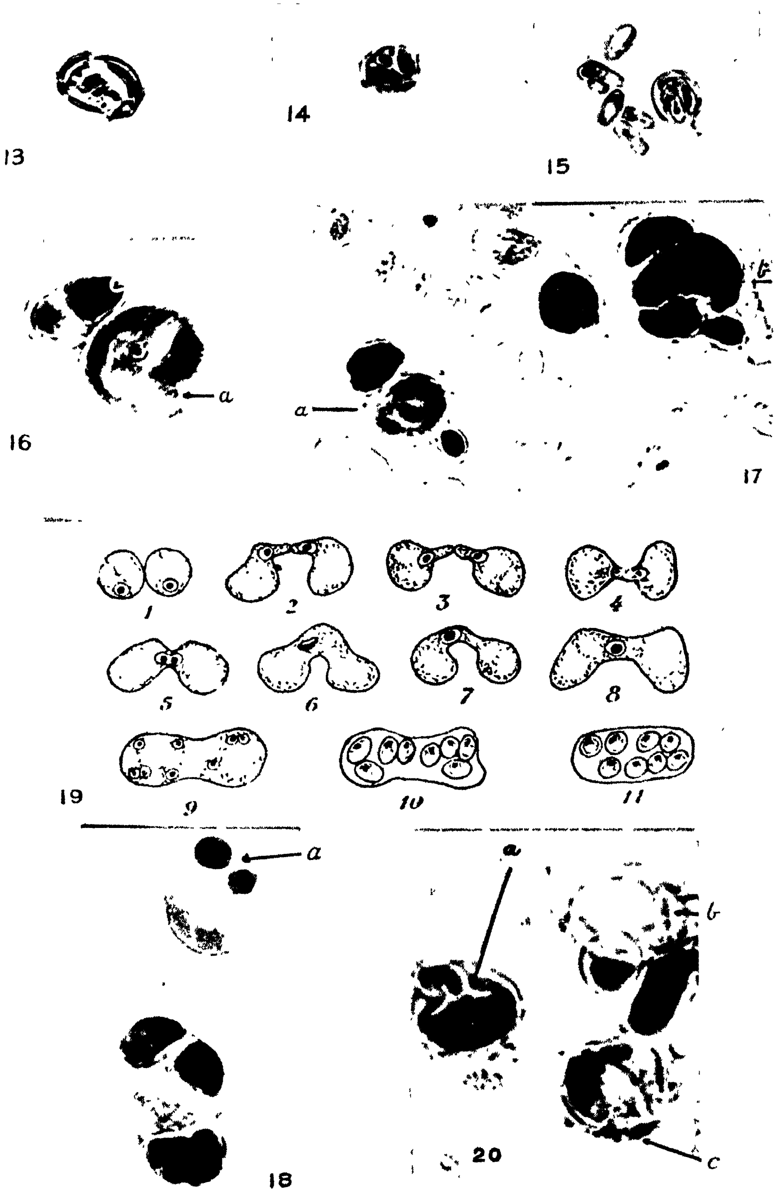
the cultures' average viability is about a month or less, but under conditions where these secondary black colonies develop the viability has been extended for as long as two and a half years.

Under these conditions the primary growth constituting the normal form of the organisms does not stain, while the intense staining of the pigmented forms is usually a safe indication of their viability. Occasionally, however, even they may undergo a progressive fatty type of degeneration as often seen in the dead normal forms, and when in this stage they are usually poorly staining and non-viable.

Here we have a condition of affairs that in its bearing on the nature of "involution forms" with bacteria is highly significant. We see that these oidia and related forms of special resistance are entirely comparable to what the bacteriologist is pleased to term involution forms. In fact if we were to look at these fungi with a low power microscope the morphologic resemblance of *itself* would suggest a pleomorphic bacterium; but the analogy extends much further than that. Just as with the bacteria we find that these pleomorphic forms of special resistance are often segregated in secondary colonies. Furthermore they are possessed of modifications in the reproductive mechanism, and—as we shall show in another paper—may be the *anlage* for the origin of variants within pure line cultures. Thus the analogy is complete, constituting rather formidable evidence for our contention *that biologically bacteria may be properly regarded as fungi which have been telescoped down into a state of existence where their life cycles, although much compressed and often abbreviated, are still not obliterated.* It would seem that it is largely in this sense that bacteria can be regarded as "simpler" forms.

ASCOSPORE FORMATION AND OTHER FORMS OF SPECIAL RESISTANCE

It is clear then that the secondary colonies of these fungi are the expression of a distinct evolutionary phase in the life history of the parasite. Since the ascospore is the "perfect stage" of the mycologist, representing as it does the culmination of the



(Mellon: Studies in Microbic Heredity)

reorganization activities, it would be natural to search for this stage in the secondary colonies, and in this our expectations were not disappointed.

It was in a secondary buff colored colony grown on non-peptone media where we found the forms about to be described. They were first observed after the culture had been grown for about two months at room temperature. In figure 11, at *a* is seen clearly an ascospore containing four normal sized, normal staining, doubly contoured forms of the parasite. The wall of the ascus at one point has almost disappeared, and in figure 12, which is the same picture taken about three hours later, one of the cells in this ascus has changed its position to a right angle and can be seen emerging from the cell. Note, too, the contraction of the ascus as the cell is forced out. This decreased size is not related to the slight change of focus necessitated; the magnification is precisely the same. The organisms were placed in a closed cell containing broth and lightly stained in thionin.

The intensely staining, diagonally located structure is an oidial filament whose details are obscured by the pigment. Note, too, that many of the cells contain circular, rather highly refringent bodies which have often been mistaken for spores, but are not doubly contoured, stain poorly if at all, and vary greatly in size. They are probably nothing but fatty granules since such structures are usually non-viable.

In figure 13 is another four-celled ascus where the definition of the individual cells is sacrificed to show the rupture points in the extraordinarily thick sclerotic capsule. In figure 14 is a ruptured ascus containing three cells; the fourth may have escaped. Figure 15 shows another ruptured ascus containing two cells. Note the frayed border of the capsule near the point for emergence of the entirely normal appearing vegetative cells. Note also that the chromatin in the free cells with one exception stains poorly.

In the large form of figure 16 is a small circular central body which may be a nucleus; a smaller one is seen at *a* in the other half of the cell, which shows a transverse line of division so frequent with these forms. In figure 17 at *a* is a similar form ap-

parently budding, as is the larger intensely staining form at *b*. Figure 18 at *a* shows early budding of a large oidial or dauern cell that may yield a circular form of the organism—the normal form being oval.

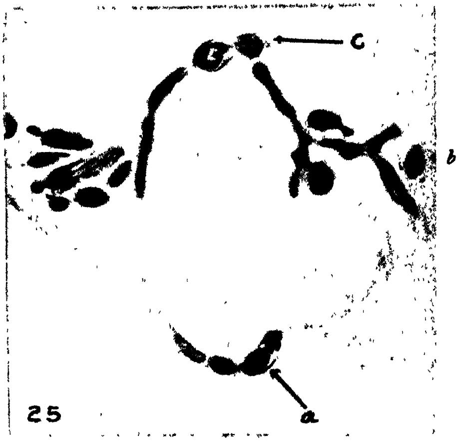
It is possible that some of these large nucleated forms are gametes inasmuch as copulation among the yeasts usually precedes the formation of the zygospore, which in turn *becomes* the ascospore. Such interpretation is entirely in harmony with the newer knowledge of the biology of yeasts as indicated in Guillermond and Tanner's book (1920). Figure 19, taken from this book, shows the nucleus and its rôle in copulation and ascus formation.

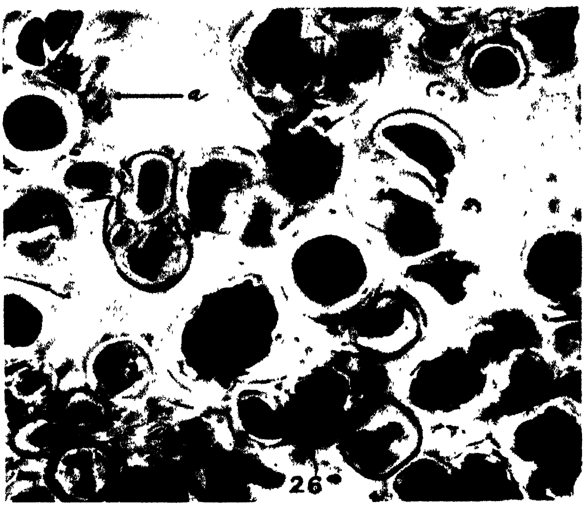
Although it is difficult to say whether the nucleus-like structure described by Ricketts (loc. cit.) is the same that we have seen, his suggestion as to its possible rôle in reproduction is pertinent. His descriptions recall the type of structure seen commonly in the "dauernzellen" which he did not recognize as such; the work of H. Will in 1895 may not have come to his attention.

GEMMATION WITH CAPSULAR DEHISCENSE

This rather interesting and unusual method of germination as suggested by figure 20 may explain in part the large number of empty cells seen in the pink or buff colored colonies. In contradistinction to the frequent non-viability of these colonies this particular one was quite viable.

In figure 20 at *a* it appears that we have a bud forcing itself through a transverse rent in the cell. Although still connected with the maternal cell, the semi-circular separation between the chromatic masses presages the formation of a transverse division wall such as characterizes an oidial filament as it springs from a maternal cell (figure 21 at *a*). At *b* of figure 20 the process appears to be somewhat further advanced since the bud, although attached to the capsular "lip" reflected from the rent, is definitely demarcated from the inner contents. The form at *c* suggests that dehiscense of the capsule is complete, the bud having escaped through the rent in the cell leaving the residual chromatin.





(Mellon: Studies in Microbic Heredity)

It is entirely possible that the new bud may completely free itself before reproducing again, or that it may remain attached for a time as indicated in figure 21. Here is an oidial filament that appears to have arisen by budding through the capsule of the older form at *a*. The capsule of the older form is undoubtedly attached to the intensely staining filament. The impression of nuclear extrusion is also given by figure 22, but the absence of a thicker wall for the extruded portion makes the form difficult of interpretation.

Figure 23 suggests an early stage of the process before rupture of the capsule. Apparently a single well developed, doubly contoured, typical yeast is preformed within the membrane of the older cell whose capsule is quite thin at this point. This form probably represents a still earlier stage of figure 10, before capsular rupture.

Vuillemin has described internal globules, absolutely analogous in appearance to yeasts, which form on the interior of the filaments of the *Endomyces albicans* (Guillermond and Tanner, 1920). He considers them as resistant forms. Such forms among the fungi are known to bud into the intercalary units of mycelium and live parasitically on their protoplasm. This preformation of adult elements within protected phases of the parasite appears to be related to the forms just described by us.

Figure 24 at *a* shows two internal globules in a bulbar dilation of a filament (Type I). This is probably one form of chlamydospore, another stage of which is seen at *a* and *c* of figure 25, also a Type I fungus. At *b* is a free one. A most striking type of resistant form is seen in figure 26; not only are the forms unusually large and intensely staining but the capsules are enormously thickened. Note size and deficient staining of "normal" forms at *a*.

It is of special interest that these forms developed in the secondary black colonies of an agar slant containing 2 per cent NaCl. Although growth on this medium was scanty, these structures were readily viable when the culture was two and one-half years old, while the poorly staining normal forms in the primary growth were not viable. The high concentration of NaCl in

this medium recalls the conditions for the development of the well known "involution forms" of the plague bacillus on NaCl agar.

Without expressing an opinion as to the real nature of such forms with *B. pestis*, it should be perfectly clear that with this fungus the only *true involution forms* are the non-viable original or so-called *normal* forms of the parasite. The fact that this is often true for similar forms with the bacteria makes it inconceivable that they should continue to be regarded as they have been in the past. Vuillemin's *Cryptococcus lithogenes* has quite similar forms morphologically and physiologically, and he has given them the name of chronispores or chlamydospores.

CONDITIONS UNDER WHICH ASCOSPORE FORMATION WAS OBSERVED

Inasmuch as the asci have been observed in the buff colored or pink types of secondary colony described, it may not be out of place to indicate roughly the conditions under which the latter were observed. Inasmuch as the media in this laboratory are routinely buffered with disodium glycerophosphate as reported by us some time ago (Mellon, 1921) this salt was usually a component.

When 2 per cent sodium chloride was added to a medium buffered to a pH of 8.0 with glycerophosphate some of these pigmented colonies were observed to occur after several weeks in the ice chest, preliminary growth having been effected at room temperature as is always the case for this strain unless otherwise stated. They have also been observed to occur on Bordet's glycerin-potato medium, though it is rare for them to appear before a few weeks at least. The essential purity of these cultures was certified by their origin from a single cell and by the fact that the secondary colonies almost invariably gave rise to the original form of the organism.

More commonly we employed a veal infusion without peptone, buffered with sodium glycerophosphate to a pH of 6.0 by

titration with HCl. Three per cent (3 per cent) of glycerin or 1 per cent of maltose was added to this agar just before use.

I have got the impression that drying of the media facilitated the occurrence of these special growth forms. This is evidenced in two ways: First, by their occurrence near the upper part or the sides of the tube where the medium was slowly evaporating, and second, by their more frequent development on a medium which had evaporated from one-fourth to one-third of its volume before being somewhat diluted by the addition of the sugar solutions above mentioned.

Glycerin usually predisposes to a raised folded type of secondary growth which maltose is not so apt to give. Although it is on the latter type of medium that ascospore formation has been most constantly observed with this strain, I do not wish to give the impression that it did not occur at times on other media. For example, it was observed on ordinary agar containing peptone and buffered with the glycerophosphate salt to neutrality.

While thus detailing in a general way the conditions under which we have observed ascospore formation to take place we appreciate nevertheless that there are certain factors having to do with the strain itself that may frustrate, or perchance facilitate, the reproduction of this or other stages in the life cycle of an organism. We are distinctly of the impression that the descendants of these specialized forms of the secondary colonies *inherit* some of the adaptability of which the forms themselves are the expression.

This is shown, first, by the relative ease with which they may recapitulate the development of secondary colonies, independently it would seem of the media; secondly, by the fact that other pure lines which have *not* been recently descended from these reorganized forms often show no tendency to form secondary colonies, even on the identical media. But these considerations begin to involve the origin of differences within pure line strains of these fungi.

Inasmuch as the next paper of the series considers the biologic origin of the mould type (Type III) from our Type II it would seem appropriate to discuss the evidence for the view just ex-

pressed in this communication. Such occurrences explain why it is not always possible to formulate an environment that will evoke the desired stage in an organism's life cycle.

DOES ASCUS FORMATION OCCUR IN TISSUES OF HUMAN CASES?

I have referred to a work of Wade and those whom he quotes, dealing with the possibility of endosporulation in the host's tissues. Although Wade states that he observed no evidence of true endosporulation the cultural results which we have obtained suggest a different interpretation of the empty shell-like figures which he finds in the tissues. In his figure 8, which is reproduced as our figure 27, one observes a large body with a thick laminated capsule, apparently not intact, and whose contents are amorphous. This capsular formation is believed by Wade and certain European observers to be the direct result of the action of the tissues and not the result of the vital activity of the parasite.

In form and size the resemblance of these tissue structures to our ascospore cases opens up the possibility of their being a true ascus from which the cells have escaped. It is conceivable, too, that they represent abortive ascus formation. That the latter does occur is brought out by our figure 8 at *b* where a form of exactly the same proportions and morphology is encountered in a culture where no true ascospores could be made out with certainty; yet we usually find them associated with the deeply pigmented oidial forms as indeed the figure shows; so this form may represent an abortive attempt at ascus formation.

In respect to the origin of morphologic forms of parasites that are believed to be the result specifically of the influence of the host's tissues, I (1919) described years ago an in vitro club formation with a streptothrix that I regarded as homologous with these well known formations with actinomyces. Latterly Bayne-Jones (1925) has verified the observations with the true actinomyces showing that club formation occurs in plain agar in the test tube.

DISCUSSION

The bearing which these observations may have on the chronicity of the disease is obvious. It is a matter of common knowledge among those who have seen cases treated clinically with potassium iodide or x-ray, or both, that lesions may seem often to disappear entirely, only to break down at the same or at a distant location. These sac-like structures occurring in the cultures are highly resistant as indicated by their increased viability. If the similar forms present in the tissues are also highly resistant the clinical recrudescences may find explanation in their subsequent germination.

The cultures indicate that the new race so derived has been physiologically rejuvenated, which permits it to make a renewed assault on the host. The ready susceptibility to destruction of young forms by therapeutic agents would contrast with the relative invulnerability of these larger forms having the thick hyaline-sclerotic capsules which may make them fast, so to speak, to agents directed against them.

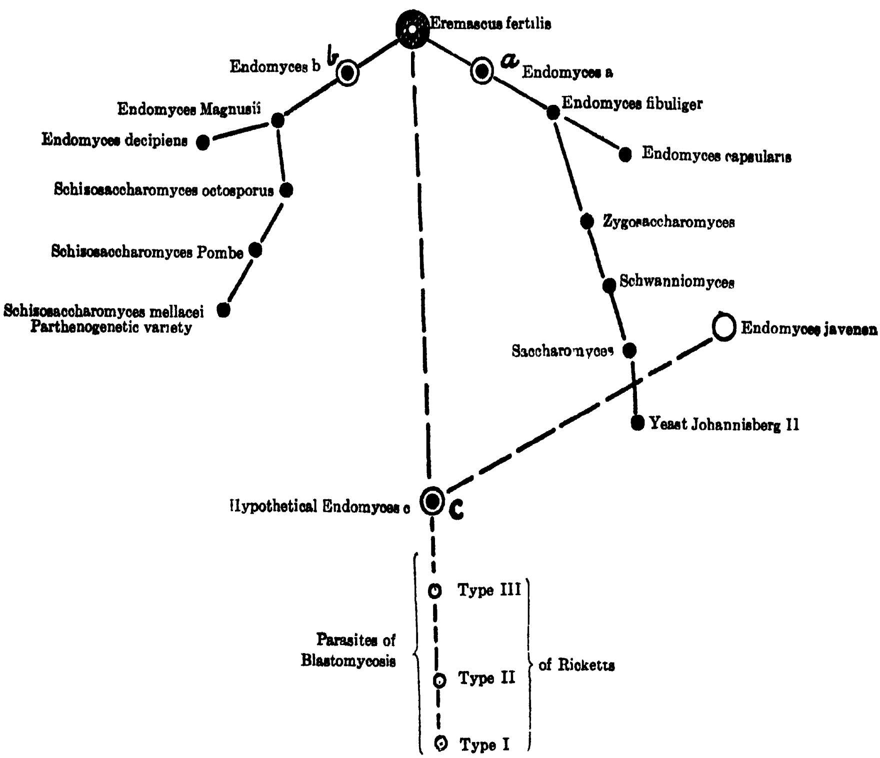
TAXONOMIC CONSIDERATIONS

What effect does the demonstration of ascospores have on the taxonomic position of these forms? An entirely comparable situation existed with the Thrush fungus which has always been regarded as an oidium until Vuillemin (quoted by Guillermond and Tanner, loc. cit.) reclaimed it for the endomyces family by finding ascospores in a certain strain. The failure of others who have since attempted to demonstrate them is attributed to the plurality of the varieties of this fungus as demonstrated by Castellani (1916) and others. Accordingly on the basis of their presence in one strain Vuillemin classes the fungus as an *endomyces* or ascomyces.

On similar grounds one seems justified in regarding the blastomycosis parasites as belonging to the ascomycetes, which would abrogate their inclusion among the oidia. Furthermore, their classification by Vuillemin as cryptococci—because no asci were observed—also becomes obsolete. Furthermore, the validity

of the oidium as a genus has gradually come into disfavor with mycologists.

According to Guillermond its general form is identical with the schizosaccharomycetes, a yeast reproducing largely by fission. Certain of our parasitic oidial forms appear to reproduce in this way (fig. 7). The diagram below taken from Guillermond and Tanner shows that the schizosaccharomyces are direct descendants of an endomyces which has an oidial phase in its life history, while the saccharomycetes or finely budding forms descend directly from an endomyces that has a budding instead of an oidial phase in its life history.



Now since the blastomycosis parasites exhibit conspicuously both budding and oidial phases arising from a mycelium that produces both asci and chlamydospores, it would appear that

they occupy an intermediate position between the lines of descent as diagramed above. The postulation of a third hypothetical endomyces *c* as shown in the diagram helps to clarify their phylogenetic relationships. The validity of such provisional arrangement is reinforced by the fact that the *Endomyces javenensis* can be successfully allocated in it.

This is a species existing in nature whose characters make its position transitional between endomyces and saccharomyces, much as the characteristics of the parasitic group make them transitional between saccharomyces and schizosaccharomyces. The conception of both these transitional groups arising from a transitional ancestor would not seem unnatural. Inasmuch as the organisms isolated from the Pacific Coast cases have not, to my knowledge, been shown to reproduce by budding it does not seem that they should be included in this group, but rather among the true molds.

CONCLUSION

1. Evidence is produced for the formation of four-celled asci with Types I and II of the parasites of blastomycosis as described by Ricketts. This *perfect stage*, according to mycologists, represents a form of endosporulation not definitely observed before.

2. This phase of the culture's development appears in our experience exclusively in the so-called secondary colonies, although all varieties of secondary colonies do not contain them. Associated with the asci are other forms of special viability such as the "dauernzellen," and oidia which may or may not be pigmented.

3. The probable occurrence of asci and related special growth forms in the tissues of the host offers plausible explanation for recrudescences of the disease after apparent cure.

4. Demonstration of ascus formation in the cultures suggests allocation of these organisms among the ascomycetes (endomyces) rather than with the oidia of Ricketts or the cryptococci of Vuillemin.

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STUDIES UPON BACTERIAL SPORES

I. THERMAL RESISTANCE AS AFFECTED BY AGE AND ENVIRONMENT

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INTRODUCTION

The resistance of bacterial spores to the destructive action of high temperatures has been a matter of general interest to bacteriologists for many years, but the significance of this resistance as a factor in practical home and industrial problems, particularly in the preservation of foods by canning, has but recently come to be appreciated.

The pioneer work of Russell (1895) thirty years ago, followed promptly by that of Prescott and Underwood (1897, 1898) established beyond question the relation of bacteria to food canning problems, and since that time numerous investigators have confirmed their conclusions and shown that successful food preservation is largely a problem of applied bacteriology. The findings of the early investigators were promptly evaluated and the processes involved in the canning of foods were modified to conform to the new information. Bacteriological investigations in this field were, however, for many years concerned primarily with the isolation and study of the microörganisms causing spoilage in canned foods, no thorough study being undertaken of the fundamental problems involved, particularly with respect to the thermal relations of bacterial spores.

Attention was forcibly drawn to this matter during the World War by heavy losses from valuable stocks of canned foods in warehouses, through spoilage, and particularly by outbreaks of

botulinus poisoning resulting from the use of insufficiently processed canned foods. Studies which these experiences initiated are still in progress.

For a thorough understanding of the problems involved in the present investigations, some familiarity with the observations of other workers upon bacterial spore resistance is essential. Sufficient space, however, is not available here for a detailed review of their reports, and so for the benefit of those who may care to look into the subject further, a condensed summary of their findings is appended at the end of this paper.

It is apparent that a number of influences are operative in determining the resistance of spores. In the reports of other workers it has been pointed out that the hydrogen-ion concentration of the medium in which the spores exist is one factor, and it has been suggested that the age of the spores may have something to do with their degree of resistance. One group of workers has made observations which indicate that possibly the amount of moisture present may be important, and that oil or other non-aqueous liquids may exert a protective influence. Others have observed that the kind of nutrient substances used in the cultivation of certain pathogenic forms apparently affects the resistance of the spores developing in the cultures, and the temperature of incubation has likewise been pointed out as an important factor.

Outside influences, however, have not been responsible for all the variations noted; for differences in resistance have been recorded where identical cultural conditions prevailed, and the opinion has been voiced that variation in resistance is an inherent property of spores which is not affected by conditions of environment.

It is seen, therefore, that the results of investigations by various workers are inconclusive, and further study of the subject is needed.[1] The matter is of immense practical importance as well as of considerable theoretical interest.

[1] Since the manuscript of this paper was completed the attention of the writer has been drawn to an important contribution to studies upon spore resistance by Esty and Williams (Jour. Infect. Dis., vol. 34, no. 5, p. 516–528), published

The problem of spore destruction is a complex one. The high resistance which many spores exhibit calls for rigorous treatment of the medium in which they are present to assure its sterilization. From the standpoint of the food canner this is a serious matter, for the processing required to destroy the troublesome spores often means injury to the appearance and quality of the canned product. Of particular significance, however, from both the practical and theoretical standpoints is the variation in resistance which they show. Not only do the spores of various species of bacteria show differences in their degree of resistance, but different strains of the same species often vary widely in this respect. Of still greater significance is the wide variation in resistance of the individual spores of a single strain. That these facts have an important bearing upon the occasional failure of hitherto successful practices in food canning can hardly be questioned; and until more is known concerning the factors which determine the resistance of bacterial spores there will always be uncertainty as to the outcome of particular canning procedures.

The investigations herein recorded were undertaken for the purpose of getting light upon certain phases of this important problem, and it is hoped that the results secured will not only be of assistance in removing much of the uncertainty that now accompanies food canning, but also help to a better understanding of the nature of bacterial spores.

EXPERIMENTAL PART

In preparation for a study of the factors affecting the variations in heat resistance of bacterial spores several matters had to be taken into consideration:

1. The selection of a suitable organism for the tests.
2. The development of a method that would assure maximum spore formation.

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just as the present investigations were being completed (May, 1924). It is a pleasure to note that although their investigations were headed in a slightly different direction, and different methods were employed in the determination of thermal death points, the results of the two pieces of work, so far as they cover the same ground, are mutually confirmatory.

3. The establishment of a definite point of departure for experiments through study of the spore cycle of the organism under observation.
4. Provision for the preparation and standardization of spore suspensions.
5. The development of a method for testing the thermal resistance of the spores.

THE ORGANISM

The organism selected for these studies was the *Bacillus mycoides*, one of the species of bacteria normally present in garden soil. It is commonly associated with the decomposition of organic matter and has frequently been isolated from canned foods. The strain of this organism used was one of 19 isolated from garden soils of 17 different states and was chosen because of the relatively high thermal resistance of its spores as shown by preliminary tests. This particular strain was isolated from Texas soil.

A number of characteristics of the *Bacillus mycoides* make it an especially favorable organism for studies of this sort: it is readily cultivated in the laboratory by ordinary laboratory technique, and grows rapidly; it forms spores readily and abundantly when proper environmental conditions exist; it has a striking and characteristic type of growth which facilitates the reading of tests and the detection of contamination; and it is non-pathogenic.

The morphological and physiological characteristics of the *Bacillus mycoides* are too well known to require consideration here. It is important to note, however, that an abundant oxygen supply is essential for spore formation, and that the optimum temperature for vegetative growth is 30°C.

MAXIMUM SPORE FORMATION

In the cultivation of *Bacillus mycoides* in the laboratory it is observed that spore formation takes place, most abundantly at least, at the surface of the medium. This being the case, provision for maximum aeration of the cultures in this work appeared necessary. Under laboratory cultivation it is also noted that this organism forms a thick felt-like scum or pellicle upon the surface

of liquid media, and a tough tenacious surface growth upon solid media, such as nutrient agar. So tough is this mass growth that it is impossible, except in very old cultures on solid media, to prepare an even suspension of the organism or its spores by ordinary agitation in liquid. To make thermal death point tests uniform suspensions of the spores are required. It was necessary, therefore, to provide for some means of separating the filaments or chains of cells at any stage in the development of the culture.

As a result of preliminary trials a method was developed which furnished ideal conditions for both the aeration of the cultures and the ready separation of the cells and spores as desired. The method was as follows:

Clean, fine quartz sand, such as is used in greenhouse experiments, was passed through a standard 40-mesh brass sieve, subjected to thorough washing, and then dried. After drying this was measured into the ordinary glass petri dishes, 15 by 100 mm. in dimensions, in 25-gram quantities, distributed to an even depth over the bottom of the plate, and then roasted in the dry air oven until completely sterile. This sand served as the substratum for the culture.

A satisfactory nutrient solution was next sought, and after repeated trials of different substances standard beef extract-peptone broth, made according to the formula recommended in the Manual of Methods for Pure Culture Study of Bacteria (1923) was selected as most suitable for the work.

In preparing these sand cultures a suspension of the spores from an old agar culture was made in a quantity of the sterilized nutrient broth, and brought to a boil in order to destroy all vegetative forms that might be present. With a sterile pipette, using aseptic precautions, just enough of this spore suspension was transferred to the sand plates exactly to saturate the sand, and the cultures thus prepared were placed in the incubator at 30°C.

It is seen that under these conditions the spores were suspended in a highly favorable nutrient medium, the fine particles of sand with their adsorbed layer of air assured maximum aeration, and the temperature of the incubator was the optimum growth temperature for this organism. Hourly examination by microscopic

technique of the grains of sand from these cultures early showed the chains of cells hugging closely the surfaces of the sand particles, and their very rapid development proved that optimum conditions for growth had been provided. Very prompt and abundant spore formation was likewise secured.

THE SPORE CYCLE

It was necessary at this point of the work to determine the length of the spore cycle of the organism under study in order that exact information might be had as to the age of the particular spores being used at any time, and especially that freshly formed spores might be subjected to thermal death point tests, so that a definite starting point for resistance studies might be established.

Accordingly, sand cultures were prepared from a suspension of spores in the manner described, and placed at 30°C. to incubate. At the end of one hour, and at hourly intervals thereafter, suspensions were prepared by shaking a small quantity of these sand cultures in tubes of sterile water. Microscopic examination was then made of these suspensions for evidence of vegetative growth and spore formation.

After preparation of specimens for microscopic examination a portion of the suspension was heated for one minute in a small tube immersed in boiling water and inoculations were made from this into nutrient broth. It was hoped in this way to determine the length of the germination period as well as to check upon the microscopic findings with respect to the length of the spore cycle. The cultural method failed to accomplish its purpose, however, as either the heating was not sufficiently long continued to destroy all vegetative cells or else the germination period of some of the spores equaled or exceeded the length of the spore cycle for other cells. The microscopic tests, however, proved satisfactory as they afforded an opportunity not only for determining the length of the spore cycle but also for observing the various stages passed through by the organism in the formation of spores.

The protocol of one of these experiments is given as follows:

Determination of length of spore cycle of Bacillus mycoïdes

Material: Sand cultures prepared by inoculating sterile sand with a suspension of the heat spores in nutrient broth.

Temperature of incubation: 30°C.

Date of test: November 26 and 27, 1923.

Microscopic findings:

TIME	INTERVAL	NOTES
	hours	
9:30 a.m.	At start	Spores only
10:30 a.m.	1	Spores only
11:30 a.m.	2	Spores only
12:30 p.m.	3	Spores only
1:30 p.m.	4	Vegetative growth, some spores
2:30 p.m.	5	Vegetative growth, some spores
3:30 p.m.	6	Vegetative growth, some spores
4:30 p.m.	7	Vegetative growth only
5:30 p.m.	8	Vegetative growth only
6:30 p.m.	9	Vegetative growth marked
7:30 p.m.	10	Vegetative growth marked
8:30 p.m.	11	Vegetative growth marked
9:30 p.m.	12	Vegetative growth abundant
10:30 p.m.	13	Vegetative growth abundant
11:30 p.m.	14	Vegetative growth abundant
12:30 a.m.	15	Vegetative growth very abundant
1:30 a.m.	16	Vegetative growth very abundant
2:30 a.m.	17	Cells show slight internal granulation
3:30 a.m.	18	Cells show marked granulation
4:30 a.m.	19	Granulation advanced
5:30 a.m.	20	Uniting of granules (advanced)
6:30 a.m.	21	Completed spores observed
7:30 a.m.	22	Abundant spores
8:30 a.m.	23	Very abundant spores
9:30 a.m.	24	Very abundant spores

It was thought that possibly the number of spores inoculated into the sterile sand in the preparation of cultures might have some influence upon the length of the spore cycle, as the accumulation of metabolic products is known to affect vegetative growth, and presumably might hasten or retard spore formation. Accordingly, other tests were made using cultures that were prepared by inoculating one set of sand plates with a full-strength

suspension of spores and another with the same diluted to one-tenth of its original strength.

The results of these comparative tests showed no perceptible differences in the progress of spore formation, the spore cycle being completed in both cases in twenty-one hours.

The length of the spore cycle among bacteria is not only of great importance, for the purposes of an investigation like the present; information in regard to this point is also necessary for an adequate application of bacteriological methods to practical problems. It has been the practice of bacteriologists for many years to sterilize certain kinds of culture media by what is known as the fractional sterilization method, which consists in heating the substance at 100°C. or less for a definite length of time on each of three successive days. With the development of home food canning the intermittent process of sterilization has come into wide use, particularly in the Southern States, and the experience of home canners, as well as of bacteriologists in the laboratory, has been that at times this method of treatment fails. An explanation for such failure is to be found in the spore cycle study which indicates that often too long an interval has been allowed to intervene between the first and second heat treatments; types of food destroying bacteria which have short spore cycles find time to return to the spore form again, and thus survive the second, and sometimes the third cooking.

PREPARATION AND STANDARDIZATION OF SPORE SUSPENSIONS

The preparation of uniform spore suspensions was made easy by the loose sand substratum. This, while it furnished optimum conditions for vegetative growth and abundant spore formation, made possible the separation of the filaments and cells so that an even and satisfactory suspension could be obtained in a very few moments by merely shaking a small quantity of the sand culture in a tube of sterile water.

In order to eliminate the possible effects of residual nutrient substances and metabolic products of the culture in the thermal death point tests the spores were washed twice with sterile distilled water, and a final suspension made of the washed spores.

This was accomplished as follows: About 20 cc. of sterile distilled water was transferred aseptically to a sterile 20 by 150 mm. culture tube. By means of a small flat-edged metal scoop, freshly flamed, a quantity of the sand culture was introduced into the tube, the cotton plug returned, and the tube agitated until a sufficient number of spores had been freed into the liquid. The resulting even suspension was then transferred by means of a sterile pipette to a sterile centrifuge tube and centrifuged at high speed to throw down the spores. The supernatant liquid was then pipetted off and more sterile water added to the tube in such a way as to bring the spores into suspension again. Centrifuging was repeated and the clear liquid pipetted away as before. This repeated washing in relatively large quantities of sterile distilled water removed from the spores all substances which might affect their thermal death points in the succeeding tests. The washed spores were then suspended again in sterile distilled water and the suspension standardized by its opacity, following the method described by Brown and Kirwan (1915).

METHOD FOR TESTING THERMAL RESISTANCE OF SPORES

For the accurate determination of thermal death points it is essential that the application of heat be instantaneous, or as nearly so as possible, in order that all may receive identical treatment. Failure in this respect may be responsible for conflicting results or may lead to erroneous conclusions. It is also highly desirable, if not absolutely necessary, that all treated spores be given an opportunity for germination, for the culturing of a small portion, such as a loopful or drop, from a treated suspension of spores is likely to give approximate results only. For these reasons it was not considered best to follow the methods of Bigelow and Esty (1919) which have hitherto been considered especially desirable for work of this sort. The tubes with which these workers made their tests were 7 mm. in internal diameter and 250 mm. long, with walls 1 mm. thick. It takes a perceptible time for heat to pass through 1 mm. of glass and still more for an equilibrium of temperature to be reached in a column of liquid 7 mm. in diameter. It was decided, therefore, that thin walled capillary

tubes should be used to contain the spore suspension to be treated, and that these should be introduced entire into the culture tubes for sterility tests.

It was realized that capillary tubes had the disadvantage of holding but small quantities of the suspension, but the fact that the number of spores in the suspension could be made as large as desired seemed to dispose of this objection.

Owing to the fact that the hydrogen-ion concentration of liquids is altered when heated in soft glass tubes, due to the dissolving out of alkalies, as shown by Esty and Cathcart (1921), "Pyrex," a very hard and insoluble glass was chosen for this work. Tubing having an internal diameter of 4 mm. was drawn out to capillaries of 1 to 1.5 mm. in internal diameter, these cut into lengths of 9 to 10 cm. and the ends sealed immediately in the flame to keep the interior sterile until needed for use.

The spore suspension having been prepared and standardized in the manner described, a quantity was transferred with aseptic precautions to a small sterile shell vial held at a convenient angle by inserting the base in a lump of modeling clay.

The capillary tubes were then charged with the spore suspension according to the following technique: Tubes sufficient in number to meet the requirements of the test were placed in a glass stender dish and covered with alcohol. As each was needed it was picked out with freshly flamed forceps, the alcohol burned off to sterilize the outside, and the sealed tips clipped off by means of a special clipping instrument freshly sterilized in the flame. One end of the tube was then dipped into the spore suspension which rose in the tube by capillary attraction. When sufficient liquid had been taken up the tube was removed, the liquid centered in the tube so as to leave a free space at each end, and the tips of the tube sealed immediately in the flame.

As rapidly as they were filled and sealed the tubes were dropped into a vessel containing cold potassium bichromate-sulphuric acid cleaning solution. This was done to kill any spores adhering to the outside of the tube which had not been destroyed at the time the tips were sealed. They were allowed to remain in this solution until all the tubes were charged. The cleaning solution

was then drained away and the tubes washed in clean cold water. When washed the tubes were transferred to a dish of fresh alcohol kept cold by placing on crushed ice. This precaution was taken to prevent any tendency of the spores to germinate, which might have affected their resistance.

The tests were made in series of fives, that is, five tubes were used in making a resistance test at each time interval.

The source of heat was an electrically heated and controlled constant temperature oil bath, fitted with a motor driven stirring device, which assured a uniform temperature throughout the bath. "Wesson" oil was the heating medium used.

To make the exposure, five of the suspension-charged tubes were removed from the cold alcohol with sterile forceps and transferred to a small aluminum holder, and the receptacle immersed in the hot oil. Both the immersion and the removal were performed as rapidly as possible and the length of exposure timed by the watch.

At the conclusion of the heat exposure the tubes were immediately transferred to a 4-ounce salt-mouth glass stoppered bottle containing fresh acetone, which dissolved off the oil. By means of sterile forceps they were then removed to fresh alcohol to keep them sterile until inoculations into nutrient media could be made. This, in most instances, was done at once, and in no case did the time exceed more than two or three minutes.

The sterility tests were made by flaming the plug and mouth of the culture tube in the usual way and then introducing the capillary tube containing the spore suspension. In preparing the capillary tubes for this inoculation they were withdrawn from the alcohol with sterile forceps and, without flaming, one sealed tip was removed with the freshly flamed clipper. The open end was then inverted over the mouth of the culture tube from which the cotton plug had been removed, and the upper tip of the capillary snipped off. At the same instant the capillary tube was released and dropped into the nutrient broth of the culture tube. The plug was then replaced, and after making certain that the contents of the capillary had been forced up into the medium by the bubble of air formed when the tube touched the medium, the culture was ready for incubation.

These operations, which may perhaps appear complicated from the description, were in reality very simple, and rapidly performed. In all cases the spores exposed to the heat treatment were placed under optimum conditions for germination within five minutes of the time the tubes were withdrawn from the oil bath.

The determination of the thermal death point consisted simply in the incubation of these cultures at 30°C., noting the point at which germination ceased, as indicated by the last positive and the first negative growths in the culture tubes.

It might seem that with so many operations entering into the tests the danger of contamination would be very great and the interpretation of results, therefore, difficult. As a matter of fact, contamination did not occur in 1 per cent of the tests and the results were clear-cut and definite. As was earlier pointed out, contamination when present was easy to detect because of the very unusual and characteristic growth of *Bacillus mycoides* in broth cultures made in this way.

This technique has the merit of assuring uniform and instantaneous exposure of all spores to the heat, and of giving to each the opportunity to germinate.

STUDY OF FACTORS AFFECTING VARIATION IN RESISTANCE OF SPORES

The relation of the hydrogen-ion concentration of the medium to the destruction of bacterial spores has already been given consideration at the hands of Buchanan and his collaborators (1918), Esty and Cathcart (1921), and Bigelow and Cathcart (1921), and it was decided, therefore to eliminate this subject from the present study.

Because so many as yet unknown elements enter into the matter of the relation of food supply, protective colloids, metabolic products, etc., to the resistance of spores it seemed desirable, in so far as possible, to eliminate these factors also from the present investigation.

The influences that might possibly affect the resistance of spores which it was decided to investigate at this time were those of the age of the spores and the environmental factors of temperature

and humidity up to the time of the tests. The plan pursued was as follows:

Sand substratum spore cultures were prepared as previously described, with the modification that sterile clay tops were substituted for the glass covers of the petri dishes. This was done to facilitate the regulation of the humidity of the cultures within the storage chambers. These cultures were incubated at 30°C. for two days to allow for maximum spore formation, as indicated by the spore cycle tests.

At the end of this period of incubation a thermal resistance test upon the young spores, twenty-four to thirty hours old, was performed to establish a point of departure in the determination of thermal resistance variations. The remaining cultures were grouped into series for storage under environmental conditions as follows:

Temperatures—ice-box (approximately 10°), 20° and 30°C. (optimum growth temperature)
Humidities—over a dehydrating agent (CaO), over carefully adjusted sulphuric acid solution (50 per cent humidity), and in water-saturated atmosphere (100 per cent humidity)

The age factor was to be studied by resistance tests made at intervals of thirty days upon each of these series of cultures.

As arranged, series of sand cultures were stored at each of the three temperatures mentioned, in humidity chambers whose atmospheres had moisture contents of approximately zero per cent, 50 per cent, and 100 per cent respectively. Resistance tests performed at thirty-day intervals upon cultures stored under these sets of conditions made possible a study of the influence of age under nine distinct situations, and corresponding studies upon the factors of temperature and humidity.

The results of these tests are presented in tables 1 to 9.

DISCUSSION OF TABLES

Age factor

That age is a factor in determining the resistance of the spores of *Bacillus mycoides* to heat is abundantly shown by the foregoing

results of thermal death time tests. In no case did the resistance of the spores remain constant throughout the period of the tests, or even approximately so. The nearest approach to constancy was observed in the results shown in table 1, but even here perceptible change occurred.

In some instances the degree of resistance increased rapidly during the early days of the test period and then dropped again;

TABLE 1

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held in a desiccator over calcium oxide in an icebox at a temperature of approximately 10°C., for from thirty to one hundred and eighty days. Exposures in these and succeeding tests made in oil bath maintained at 100°C. Suspension in these and succeeding tests contained 150 million spores per cubic centimeter

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	+++++	No test	+++++	+++++	+++++
1	+++−−	+++−−	+++++	+++++	+++++	+++++	+++++
1½	+−−−−	−−−−−	++++0	No test	+++++	++−−0	++−−−
2	−−−−−	−−−−−	++−−−	++−−−	−−−−−	+−−−−	+−−−−
3	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	+−−−−	−−−−−
4	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
5	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−

Note: Signs used apply similarly to all succeeding tables.

+ indicates positive growth in culture tube following exposure to heat of the spores under test.

− indicates no growth in culture tube.

0 indicates that the test was lost by accident.

It is seen that in a dry, cold environment there was but slight change in resistance of the spores during the entire test period, though there took place a slight increase after the first thirty days of storage.

later to be followed by another increase. The degree of resistance attained in a number of cases was two or three times as great as that of the young spores.

With several series the resistance curve rose abruptly during the first thirty days and then became horizontal, or practically so. Several showed a gradual increase in the resistance of the spores for as long as sixty days.

Temperature factor

Glancing back over the tables it will be noted that in every case, regardless of humidity, and almost without regard to the age of the spores, the greatest resistance was developed at the temperature of 20°C., indicating that a temperature somewhat

TABLE 2

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held in a dessicator over calcium oxide at a temperature of 20°C., for from thirty to one hundred and eighty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	No test	No test	+++++	+++++	+++++
1	+++−−	+++++	No test	+++++	+++++	+++++	+++++
1½	+−−−−	+++++	No test	No test	+++++	+++++	+++++
2	−−−−−	+++++	+++++	+++++	+++++	+++++	+++++
3	−−−−−	+++++	+++++	++++−	++++−	+++++	+++++
4	−−−−−	+++−−	++++−	−−−−−	−−−−−	++−−−	++++0
5	−−−−−	+−−−−	++++−	−−−−−	−−−−−	+−−−−	+++−0
6	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	+−−−−
7	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
8	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−

When held in a very dry atmosphere and at a moderate temperature marked changes occurred in the thermal resistance of the spores. Within the first thirty days of storage the thermal death time rose from two to six minutes, indicating a threefold increase in resistance over that of the original spores. This degree of resistance was maintained for another thirty days, and then showed a decided decline. At one hundred fifty days the resistance began to increase again, and at one hundred and eighty days was highest during the period of the tests. The significance of this fluctuation is not known.

below the optimum for vegetative growth, and in most cases well above the minimum, seems to be most favorable for change in spore resistance, at least in the case of *Bacillus mycoides.*

Humidity factor

To evaluate the direct influence of humidity upon change in spore resistance is extremely difficult if not impossible. In com-

TABLE 3

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held in a dessicator over calcium oxide at a temperature of 30°C., for from thirty to one hundred and eighty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	No test	No test	+++++	+++++	+++++
1	+++−−	+++++	+++++	+++++	+++++	++++0	+++++
1½	+−−−−	+++++	No test	No test	No test	+++++	++−−−
2	−−−−−	+++++	+++++	+++++	+++++	++++−	−−−−−
3	−−−−−	−−−−−	+−−−−	−−−−−	++−−−	−−−−−	−−−−−
4	−−−−−	−−−−−	−−−−−	−−−−−	+−−−0	−−−−−	−−−−−
5	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−

In a dry but warm atmosphere (temperature optimum for vegetative growth) it is seen that a decided increase in resistance to heat developed in the spores during the first thirty days. This increase, while not as great as in those held at 20°C., was never-the-less marked, and was maintained for five months without marked alterations. At the one hundred and eighty-day test the resistance had fallen back to its original position, or essentially so.

TABLE 4

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held at icebox temperature (approximately 10°C.) in an atmosphere having a 50 per cent humidity, for from thirty to one hundred and eighty days. Exposures made in oil bath maintained at 100°C

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	No test	No test	+++++	+++++	+++++
1	+++−−	+++++	+++++	+++++	+++++	+++++	++++N
1½	+−−−−	+++++	No test	No test	+++++	+++++	+++++
2	−−−−−	+++++	+−−−−	+++++	+++++	+++++	+++++
3	−−−−−	++++−	−−−−−	+++++	+++−−	+++++	+++++
4	−−−−−	−−−−−	−−−−−	−−−−−	++−−−	+++−−	−−−−−
5	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	++−−−	−−−−−
6	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−

In a cold atmosphere, half saturated with moisture the spores showed a prompt and marked increase in resistance. In thirty days the degree of resistance had doubled. At the sixty-day period there was an apparent decline, but the results of the succeeding tests indicate that some uncontrolled factor influenced the results of the sixty-day tests. The highest resistance was reached at the time of the one hundred and fifty-day tests.

bination with other factors, such as temperature, it is observed to be very important, as may be seen in the case of the series of spore cultures held at the temperature of the ice-box. It is apparent that the lack of moisture had a direct restraining influence upon the change in resistance of spores held over calcium oxide in the chamber at ice-box temperature as shown in table 1. Here it will be noted that no increase occurred during the first thirty

TABLE 5

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held at a temperature of 20°C. in an atmosphere having a humidity of 50 per cent, for from thirty to one hundred and eighty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	No test	No test	+++++	+++++	++++0
1	+++−−	+++++	No test	+++++	++++0	++++0	+++++
1½	+−−−−	+++++	No test	No test	+++++	+++++	+++++
2	−−−−−	+++++	+++++	+++++	+++++	+++++	+++++
3	−−−−−	+++++	++++0	+++++	+++++	+++++	+++++
4	−−−−−	++000	+++−−	+−−−−	++++−	−−−−−	++++−
5	−−−−−	++−−−	−−−−−	−−−−−	−−−−−	−−−−−	++−−−
6	−−−−−	+−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
7	−−−−−	++−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
8	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
9	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
10	−−−−−	−−−−−	−−−−−	−−−−	−−−−−	−−−−−	−−−−−

Under conditions of medium temperature and humidity the spores showed marked increase in resistance. At thirty days evidences of an especially high resistance are noted in the case of some spores. The high degree of resistance attained for the majority of the spores was retained throughout the period of the tests.

days, although spores held at ice-box temperature but in atmospheres having humidity values of 50 and 100 per cent respectively showed a very marked increase in resistance during this period.

On the other hand, high humidity combined with low temperature resulted in as great changes as those which developed under either high humidity-optimum growth temperature or low humidity-optimum growth temperature conditions.

TABLE 6

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held at a temperature of 30°C. in an atmosphere having an humidity of 50 per cent, for from thirty to one hundred and eighty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	No test	No test	+++++	+++++	++++0
1	+++−−	+++++	+++++	+++++	+++++	+++++	++++0
1½	+−−−−	+++++	No test	No test	+++++	+++++	+++−−
2	−−−−−	+++++	+++++	+++++	+++++	+++++	+−−−−
3	−−−−−	++++0	+++++	+++++	+−−−−	++++−	−−−−−
4	−−−−−	++−−−	+++−−	+−−−−	−−−−−	++++−	++−−−
5	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
6	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
7	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−

In a warm, moderately humid environment the spores, as shown by these results, developed a pronounced increase in resistance within the first thirty days. Some irregularity in the shape of the thermal death time curve is noted, and at the time of the one hundred and eighty-day tests the resistance seems to have decreased considerably.

TABLE 7

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held at the temperature of the icebox (approximately 10°C.) in an atmosphere having a humidity of 100 per cent, for from thirty to one hundred and fifty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	No test	No test	No test	+++++	
1	+++−−	+++++	+++++	+++++	+++++	+++++	No material for 180-day tests
1½	+−−−−	++++0	No test	No test	+++00	+++++	
2	−−−−−	++++−	+++++	+++++	+++++	+++++	
3	−−−−−	+++−−	+++++	++++−	++−−−	+++++	
4	−−−−−	+−−−−	++−−−	−−−−−	−−−−−	++−−−	
5	−−−−−	−−−−−	+−−−−	−−−−−	−−−−−	−−−−−	
7	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	

In a cold, moisture-saturated atmosphere the resistance of these spores continued to increase steadily for sixty days. After six months of storage the resistance was still more than twice that of spores twenty-four to thirty hours old.

TABLE 8

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held at a temperature of 20°C. in an atmosphere having an humidity of 100 per cent, for from thirty to one hundred and eighty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	++++0	No test	No test	+++++	+++++	+++++
1	+++−−	+++++	+++++	+++++	+++++	+++++	+++++
1½	+−−−−	+++++	No test	No test	+++++	+++++	+++++
2	−−−−−	+++++	+++++	+++++	+++++	+++++	+++++
3	−−−−−	+++++	+++++	+++++	+++++	+++++	+++++
4	−−−−−	++++−	++++0	+++−0	++++−	++++−	+++++
5	−−−−−	−−−−−	++++−	−−−−−	+++++	+−−−0	++−−−
6	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
7	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−

When held in a moderately warm, moisture-saturated environment the resistance of the spores increased in the same fashion as shown in table 7, but to a slightly greater degree, and the high degree of resistance attained was still retained at the time of the one hundred and eighty-day tests.

TABLE 9

Showing the results of thermal resistance tests upon the spores of Bacillus mycoides held at a temperature of 30°C. in an atmosphere having an humidity of 100 per cent, for from thirty to one hundred and eighty days. Exposures made in oil bath maintained at 100°C.

LENGTH OF EXPOSURE	AGE OF SPORES						
	24–30 hours	30 days	60 days	90 days	120 days	150 days	180 days
minutes							
½	+++++	+++++	No test	No test	+++++	+++++	+++++
1	+++−−	+++++	+++++	+++++	+++++	++++0	+++++
1½	+−−−−	+++++	No test	No test	+++++	+++++	+++−−
2	−−−−−	+++++	+++++	+−−−−	+++++	−−−−−	+−−−−
3	−−−−−	++++−	++−−−	−−−−−	+++++	−−−−−	−−−−−
4	−−−−−	−−−−−	−−−−−	−−−−−	++++−	−−−−−	−−−−−
5	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−
6	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−	−−−−−

With maximum moisture, and optimum growth temperature supplied the spores of this series showed considerable variation in their resistance. The thermal death time curve was quite irregular.

GENERAL DISCUSSION

Having now observed the alteration in thermal resistance of bacterial spores under different conditions as regards age, temperature and humidity, we come to ask ourselves the reasons for these changes.

For many years bacteriologists have held the opinion that spore formation among bacteria represents the response of the organism to an unfavorable environment, and that the spore once formed remains dormant or inactive until a return of conditions favorable for vegetative growth.

That abundant spore formation takes place at temperatures best adapted for vegetative growth, among some species of bacteria at least, has been often observed and has been shown again in the present work with *Bacillus mycoides*. Unfavorable temperature conditions, therefore, do not determine spore formation. Insufficient food supply cannot be the controlling factor because spore formation and vegetative growth proceed side by side. Our own experiments, earlier mentioned, indicate also that the concentration of metabolic products cannot be the determining influence, for spore formation took place as quickly in a dilute suspension of organisms as in one containing ten times as many. Absence of moisture is not responsible for the formation of spores, for every bacteriologist knows that spores form in liquid as well as on solid media.

If, then, neither food supply, moisture, temperature, nor metabolic products which are the factors commonly supposed to affect bacterial activities, control spore formation, may we not look upon this phenomenon of spore formation as one of the normal phases in the ordinary existence of at least certain of the bacteria, which proves of vital importance to the species in that it enables some individuals to survive adverse conditions? May we not look upon the spore, from the standpoint of the bacteria, as a most fortunate provision against adversity rather than as a product of it?

This idea has been furthered by the analysis of the present experimental findings which prove that spores rarely, if ever, are dormant. Increase in resistance under a wide range of conditions

has been observed. This change has been found to take place most rapidly under conditions apparently not the most likely to injure the vitality of the cell but rather under what might be termed "temperate" conditions—conditions suited to positive but rather sluggish vital activities normal to the spore form.

Plant physiologists and plant chemists have come to know that the seed which used to be considered dormant is in reality undergoing fundamental internal alterations even though the change cannot be detected from its external appearance. In like manner also, tubers and other storage organs of plants which during the winter period have been supposed to be in a dormant condition have been found to have undergone great changes internally.

We are coming, therefore, to a realization that strict dormancy among living forms rarely, if ever, exists and that what has been interpreted as such is merely a retardation in the rate of vital activities.

In the light of evidence at hand it seems reasonable to conclude that variations in resistance of bacterial spores under differing environmental conditions are the results of normal spore transformations which are retarded by some and possibly accelerated by other conditions of environment.

The results of the present investigation are of general scientific interest and of practical significance.

That virulence among pathogenic bacteria may be altered in either direction by modifications in cultural practices has long been known. That virus once formed may be weakened by certain storage treatments, as is done in the preparation of the attenuated virus of rabies for the Pasteur treatment, has likewise long been known and put to practical use in the practice of human and of veterinary medicine. It has been learned also that under certain conditions of cultivation a spore-forming organism may be prevented from forming spores normally, or if doing so, may be made to form spores possessing different degrees of resistance.

The assumption has been that once a spore has been formed its resistance is a fixed property. That this idea must be abandoned seems evident, for what has hitherto been looked upon as a constant has been shown to be a variable.

The results of the present study are of practical significance in two fields: First, that which is concerned with disinfection and disinfectants; and the second, that which involves food preservation.

A few only of the disease-producing bacteria are of spore forming types, but those which possess this property are extremely dangerous. It is of importance, therefore, that all methods of disinfection and all disinfectants be selected and applied in such a way as to take into account not only the variations in the resistance of different strains of these organisms but also the changes in resistance almost certainly possible in all spores.

Of particular importance is this matter as it applies to the testing and standardization of chemical disinfectants. Germicidal values are determined, in many instances at least, by the ability of the substance in different dilutions to destroy the spores of *Bacillus anthracis* or other pathogenic spore-forming bacteria. It is absolutely essential for safety that the resistance of the spores used in these tests be as uniform as possible, and the alterations in resistance occurring under storage as well as under cultural conditions must be taken into account.

The significance of these findings as they concern food preservation lies in the importance which thermal death points of bacterial spores have in determining the temperatures and time periods of the processing, or cooking of the food in the can.

The canning industry is rapidly getting away from rule-of-thumb practices and more and more is basing its operations upon the findings of research laboratory workers. The rate of heat penetration into the cans of food during the processing has been extensively studied; the relations of the character of the food and the character of the pack to ease of sterilization have been investigated; bacteria causing food spoilage have been isolated and subjected to careful observation; and the thermal death points of numerous highly resistant spore-forming types have been determined. Upon the basis of these findings new processing schedules have been developed and the results of careful laboratory experiments are being applied in the industry.

This has been done under the assumption that the thermal

death points observed for particular organisms represented fixed values. From the present findings we are led to conclude that these values are not constant, and unless a considerable margin of safety is provided for in the standardization of food processing schedules the new practices, at times, may be expected to fail. In determining the thermal death points that are to serve as the basis of new processing schedules bacteriologists must be as certain as possible that the resistance shown by the test spores represents the highest degree attainable by them.

SUMMARY

The object of the present investigation was to throw light upon the important subject of thermal resistance of bacterial spores, with special reference to changes in resistance which have been observed to occur, and which it is thought may have an important bearing upon the problem of food preservation.

The organism selected for the study was the *Bacillus mycoides* which, because of its cultural and physiological characteristics, is particularly well adapted to the work.

The experiments were confined primarily to a determination of the influence of age and of the environmental factors of temperature and humidity upon variations in thermal resistance of spores, and data were obtained at thirty-day intervals upon spores stored under nine different sets of environmental conditions. A study of the spore cycle of the organism under test; the development of special cultural methods in order to provide for maximum spore formation and the preparation of satisfactory spore suspensions; and the development of a more satisfactory technique for making the resistance tests, were necessary parts of the investigation.

The results of the tests may be summarized briefly as follows:

1. In a cold, dry environment there was practically no change in the resistance of the spores during the first thirty days of storage, but a small increase was manifest by the time of the sixty-day tests which was maintained for the remainder of the test period.

2. When held in a very dry atmosphere and at a moderate temperature marked changes occurred. Within the first thirty days of storage the thermal death time rose from two to six minutes, indicating a threefold increase in resistance over that of the original spores. This degree of resistance was maintained for another thirty days, and then showed a decided decline. At one hundred and fifty days the resistance began to increase again, and at one hundred and eighty days was at a maximum point. The significance of this fluctuation is not known.

3. In a dry but warm atmosphere (temperature optimum for vegetative growth) a decided increase in resistance to heat developed in the spores during the first thirty days. This increase, while not as great as in those held at 20°C., was marked, and was maintained for five months without material alterations. At the time of the one hundred and eighty-day tests the resistance had fallen back to nearly its original position.

4. In a cold atmosphere, half saturated with moisture the spores developed a prompt and marked increase in resistance, the degree of resistance doubling within the first thirty days. The highest point in this series was reached at the time of the one hundred and fifty-day tests.

5. A pronounced increase in resistance resulted when the spores were held under conditions of medium temperature and humidity. An especially high resistance was shown by some of the spores at the time of the thirty-day tests and a considerably increased resistance for the majority of the spores was not only attained but also retained throughout the period of the tests.

6. In a warm, moderately humid environment the spores developed a marked increase in resistance within the first thirty days. The death time curve for the entire period of the tests, however, was somewhat irregular.

7. In a cold, moisture-saturated atmosphere the resistance of the spores continued to increase steadily for sixty days, and after six months of storage was still more than twice that of spores twenty-four to thirty hours old.

8. When held in a moderately warm, moisture-saturated environment the resistance of the spores increased in the same

fashion as the preceding but was slightly less pronounced. The high degree attained, however, was still retained at the time of the one hundred and eighty-day tests.

9. With maximum moisture and optimum growth temperature supplied considerable variation in resistance was noted. The thermal death time curve was quite irregular.

CONCLUSIONS

A careful analysis of the experimental data presented leads to the following conclusions:

1. The bacterial spore is not dormant under ordinary conditions, as has commonly been supposed, but is instead sluggishly active.
2. The resistance of spores to heat is not a fixed property but a variable one, the degree of resistance being influenced by age, the temperature and humidity of the environment, and possibly other factors.
3. The highest resistance to heat develops under conditions of moderate temperature and humidity, and is probably reached by the time the spores are sixty days old. Spores of different species of bacteria may be expected to vary somewhat in this respect.
4. Change in resistance takes place most slowly when spores are dry and cold, but low temperature accompanied by high humidity results in the development of a high degree of resistance.
5. In determining the thermal death points of spores that are to serve as the basis of processing schedules for canned foods the bacteriologist must take into account the change in resistance of spores under various conditions, and be as certain as possible that the resistance shown by the test spores represents the highest degree attainable by them.

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APPENDIX

The following is a condensed résumé of the literature bearing upon the subject of bacterial spore resistance:

1888 Globig (1888) found that spores of the "potato bacillus" withstood exposure in streaming steam at 100°C. for 5 to 6½ hours; and in superheated steam at 113 to 116°C. for 25 minutes; at 112 to 123°C. for 10 minutes; at 126°C. for 3 minutes; and at 127°C. for 2 minutes. Death instantaneous at 130°C.

1889 Esmarch (1889) found that resistance of spores of *B. anthracis* was affected by nutrient medium used.

Geppert (1889) reported variation in resistance of spores of *B. anthracis* even when grown under identical cultural conditions.

Fraenkel (1889) confirmed the findings of Geppert (1889) and concluded that variation in resistance is an inherent property of different strains and not affected by conditions of environment.

1895 Miquel and Zattraye (1895) found that moist spores of *B. subtilis* in cultures 3 to 4 weeks old survived exposure for 15 minutes at 104.4 to 108.3°C.; 30 minutes at 102.8 to 106.3°C.; 1 hour at 102.9 to 104.7°C.; and 4 hours at 99.7 to 100.1°C.

Christen (1895) described organisms whose spores resisted 100°C. for periods of 4 to 16 hours; 105°C. for 1 to 4 hours; 110°C. for 1 to 2 hours; 115 to 116°C. for ½ to 2 hours; and 130°C. for 5 minutes.

1896 Cambier (1896) found that spores of soil organisms exposed to dry heat survived after 3½ hours treatment at 110.7°C., but were killed in 1½ hours at 124°C.; and in 15 minutes at 138°C. In air-dry soil the spore survived 111°C. for 4½ hours; 136.9°C. for 3 hours; 156.5°C. for 2 hours; and 180°C. for 35 minutes. All were destroyed in 5 minutes at 200°C.

1897 Kronig and Paul (1897) confirmed the findings of Geppert (1889).

Van Ermengem (1897) reported that the spores of *B. botulinus* were only slightly resistant to heat, being destroyed in 1 hour at 80°C.

1899 Weil (1899) confirmed the work of Esmarch (1889) and noted that temperature of incubation appeared to affect resistance of spores.

1902 von Wahl (1902) found 2 hours insufficient time to sterilize carrots, asparagus, and peas packed in glass containers when exposed to streaming steam at 100°C. Spores of an organism in carrots survived 3½ hours processing in water at 100°C.

1904 Neide (1904) described 3 organisms the thermal death points of whose spores varied from 15 to 38 minutes at 100°C.

1905 Blau (1905) reported the thermal death times for spores of various bacteria exposed to temperature of boiling water as follows: *B. subtilis*, 175–180 minutes; *B. robustus*, 450 to 480 minutes; *B. calidus*, 450 to 480 minutes; *B. cylindricus*, 1140 to 1200 minutes; and *B. tostus*, 1140 to 1200 minutes.

1908 von Hibler (1908) secured constancy in the resistance of spores of *B. anthracis* by using cultures that were "neither too old nor too young." Acidity and long storage in incubator found to affect resistance of spores.

1915 Shanly (1915) studied heat resistance of spores of 23 different species. The least resistant, *B. cereus*, survived exposure to 75°C. for 1 hour but was

killed was 80°C. for same period. Others, identified only by numbers, survived after 1 hour at 100°C. Variation in resistance of different strains of same species noted.

1918 Buchanan, Thompson, Orr and Bruett (1918) gave particular attention to effect of preliminary canning operations upon thermal death points. They also determined the relation of the hydrogen-ion concentration of the product to heat resistance of bacteria and their spores.

1919 Bruett (1919) found that scalding followed by chilling is not an effective means of spore destruction.

Burke (1919) reported that while exposure to a temperature of 100°C. may not kill spores of *B. botulinus* their vitality is weakened so that germination is delayed. Spores of this organism survived 3½ hours boiling at 100°C., and 5 hours boiling was considered insufficient to sterilize. Fractional sterilization, because of delayed germination, was held of doubtful value.

Normington (1919) in studies of organisms isolated from cold-packed canned peas found that all withstood 10 to 15 pounds steam pressure in the autoclave for 10 to 20 minutes.

Bigelow and Esty (1919) developed improved method for determining spore resistance. Spores of one strain destroyed only after 16 hours boiling at 100°C.; 100 minutes at 110°C.; 50 minutes at 115°C.; 10 minutes at 120°C.; and 4 minutes at 125°C. Initial number of spores and H-ion concentration of medium were found to affect the time required to sterilize.

Esty and Williams (1919) reported resistance tests upon organisms isolated from canned foods. Considerable variation was noted in resistance, ranging from 1½ to 17 hours at 100°C. True thermophiles were found most resistant.

Thom, Edmondson and Giltner (1919) found spores of *B. botulinus* from canned asparagus survived steaming at 116°C. for 15 minutes, and 100°C. for 1 hour.

Dickson, Burke and Ward (1919) reported upon 8 strains of *B. botulinus*. All survived 3 hours heating at 90°C.; 7 of them 3 hours at 95°C.; and 6 of them 2 hours at 100°C. Addition of 5 per cent of lemon juice to meduim did not prevent growth or formation of toxin but lowered the death point of spores. Spores treated in presence of animal and vegetable protein.

1920 Bigelow and Esty (1920) studied relationship of temperature and time to spore destruction in thermophiles from spoiled canned foods. Destruction of spores in one culture as follows: 1320 minutes in boiling water at 100°C.; 690 minutes at 105°; 225 minutes at 110°; 84 minutes at 115°; 23 minutes at 120°; 8 minutes at 125°; 3.5 minutes at 130°; 1.5 minutes at 135°; and 1 minute at 140°C. Nineteen thermophiles studied showed same time-temperature relationships.

Fenger, Cram and Rudnick (1920) found thermal death points of five organisms isolated from ligatures and sutures, when heated in non-aqueous liquids, to lie between 150 and 160°C. for 1 hour.

Donk (1920) found that spores of a thermophile isolated from canned corn required 17 hours to kill when heated in corn broth at 100°C. with 12,500

spores per 1 cc.; and 11 minutes when heated at 120°C. with 50,000 spores per 1 cc.

1921 Weiss (1921) found that free spores of *B. botulinus* were killed within five hours at 100°C.; 40 minutes at 105°C.; and 6 minutes at 120°C. Young moist spores, more resistant than old, and spores 1 month old found three times as resistant as those 5 months old. More resistant individuals changed more rapidly than less resistant ones. Thermal resistance of emulsions of young spores increased as concentration of emulsion increased. Sodium chloride lowered thermal resistance. Increase in H-ion concentration on acid side of neutrality, and of hydroxyl-ions on alkaline side lowered resistance of spores. Work on spores of *B. botulinus*. In another paper same author (1921) showed relation of H-ion concentration, consistency of food material, concentration of sirup present, etc., to thermal death point of *B. botulinus;* acid foods requiring less severe treatment to sterilize than non-acid substances.

Bigelow (1921) gave the thermal death times of 15 typical thermophiles when exposed to different temperatures as follows: At 100°C., between 788 and 834 minutes; at 105°, between 383 and 405 minutes; at 110°, between 117 and 122 minutes; at 115°, between 40 and 44 minutes; at 120°, between 11 and 12 minutes; at 125°, between 3.9 and 4.6 minutes; at 130°, between 1.7 and 2.2 minutes; at 135°, between 0.7 and 0.9 minutes; and at 140°C., between 0.6 and 0.9 minutes.

1922 Dickson and his associates (1922) noted marked variations in resistance of spores of *B. botulinus*. Tests on 40 strains made at 100°C. showed survival period varying from thirty minutes to six hours. About 95 per cent spores were destroyed in short time, some of remainder highly resistant. Spores from old cultures more resistant than those from young, and resistance greater in neutral media than in acid of alkaline media. Noted delay in germination, in one case for 330 days.

Esty and Meyer (1922) found spores of *B. botulinus* more resistant than practically any other anaerobe, requiring 4 minutes at 120°C. or 330 minutes at 100°C. to kill. Spores in juices of 17 different kinds of canned foods showed variation in resistance of from less than 10 minutes to 230 minutes at 100°C.

Tanner and Dack (1922) studied resistance of spores of *B. botulinus* to dry heat. Spores survived 110°C. after 2 hours; 140°C. from 15 to 60 minutes; and 160 to 180°C. from 5 to 15 minutes.

1923 Esty (1923) found resistance of 112 strains of *B. botulinus* to vary from 3 to 75 minutes at 105°C. Maximum resistance given as 330 minutes at 100°C.; 110 minutes at 105°C.; 33 minutes at 110°C.; 11 minutes at 115°C. and 4 minutes at 120°C. Greater resistance shown in heavy suspensions than in light.

Wyant and Tweed (1923) found that for 8 organisms isolated from "flat-sour" canned peas sterilization was accomplished at above 80°C. and below 110°C. after 10 minutes heating.

Tanner and McCrea (1923) reported resistance of spores of *B. botulinus* sealed in tubes exhausted to 17 mm. Spores destroyed within 5 hours at 100°C.; 2 hours at 105°C.; 1½ hours at 110°C.; 40 minutes at 115°C.

and 10 minutes at 120°C. Longer time required to destroy spores of same age in open tubes than in tubes exhausted and sealed.

1924 Esty (1924) made inoculation experiments upon peas and corn using suspensions containing different numbers of spores. Showed that spoilage in canned product was materially increased when number of spores was increased, due to especially resistant individual spores in suspensions.

OBSERVATIONS ON "PIN POINT COLONY" ORGANISMS IN THE BALTIMORE MILK SUPPLY

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INTRODUCTION AND LITERATURE

It seems to be quite generally agreed that the "pin point colonies" which occasionally appear on plain agar plates inoculated with diluted milk for the purpose of estimating the number of organisms present may be due to a variety of causes.

Yates (1923) suggested that since the trouble was first recognized, coincident with the change in the method of adjusting the reaction of culture media and the more or less general use of chlorine solutions in dairies and on farms, there might possibly be a connection between one or both of these procedures and "pin point colonies."

Ayers and Johnson (1924) found the appearance of these colonies from pasteurized milk from one milk plant to be due ts a thermophilic organism which they named *Lactobacillus thermophyles*. They also suggested that crowded plates, the reaction of the medium and heat resistant non-thermophilic bacteria might be possible causes.

Cooledge (1924) by a special technique isolated alkali producing thermophilic bacteria from milk and found that these organisms when present in great numbers overcame the unfavorable reaction of the medium and developed as typical pin point colonies.

Harding and his collaborators (1924) have demonstrated that there are present in all classes of milk organisms which will grow at the pasteurizing temperature. We are not sure whether these organisms produced pin point colonies or not.

OBSERVATIONAL AND EXPERIMENTAL

For a little over two years we have observed in connection with our routine milk control counts that we believe to be typical cases of "pin point colonies." What we call typical cases are not due to crowded plates, for counts made from low dilutions (in so far as they can be counted at all because of their extreme minuteness) are much higher than those from higher dilutions. This would not be true if the "pin point colonies" were due to crowding. They are not due to differences in the reaction of the culture medium in so far as these differences exist prior to incubation. However, in this connection it must be borne in mind that the reaction of the medium in the plates after incubation is rarely the same as it was before incubation. Due to the activity of the developing organisms it is practically always changed. Usually the low dilutions become more alkaline than the higher dilutions, although there may be a change in the acid direction.

Late in the year 1922 we became convinced of the typical appearance of these plates. In order to report our findings as accurately as possible we estimated the number of colonies as best we could and reported the counts with the notation "pin point colonies." This signified that there were at least as many organisms present as reported and probably a great many more. This procedure has been in operation for a little over two years.

It has been our general observation that these cases of "typical pin point colonies" were much more numerous in the winter. In order to establish our observation in this regard we went over our records and calculated the percentage of the total number of examinations made each month which had shown "typical pin point colonies." This was done for each of the three main classes of milk which we analyze. Figure 1 shows the curves constructed ed from these figures for the last two years.

In order to determine if possible the organism or organisms producing these colonies we collected cultures from typical plates over a period of two months (December 15, 1923 to February 15, 1924). One or two colonies from each plate were fished under the microscope and inoculated into litmus milk. These cultures

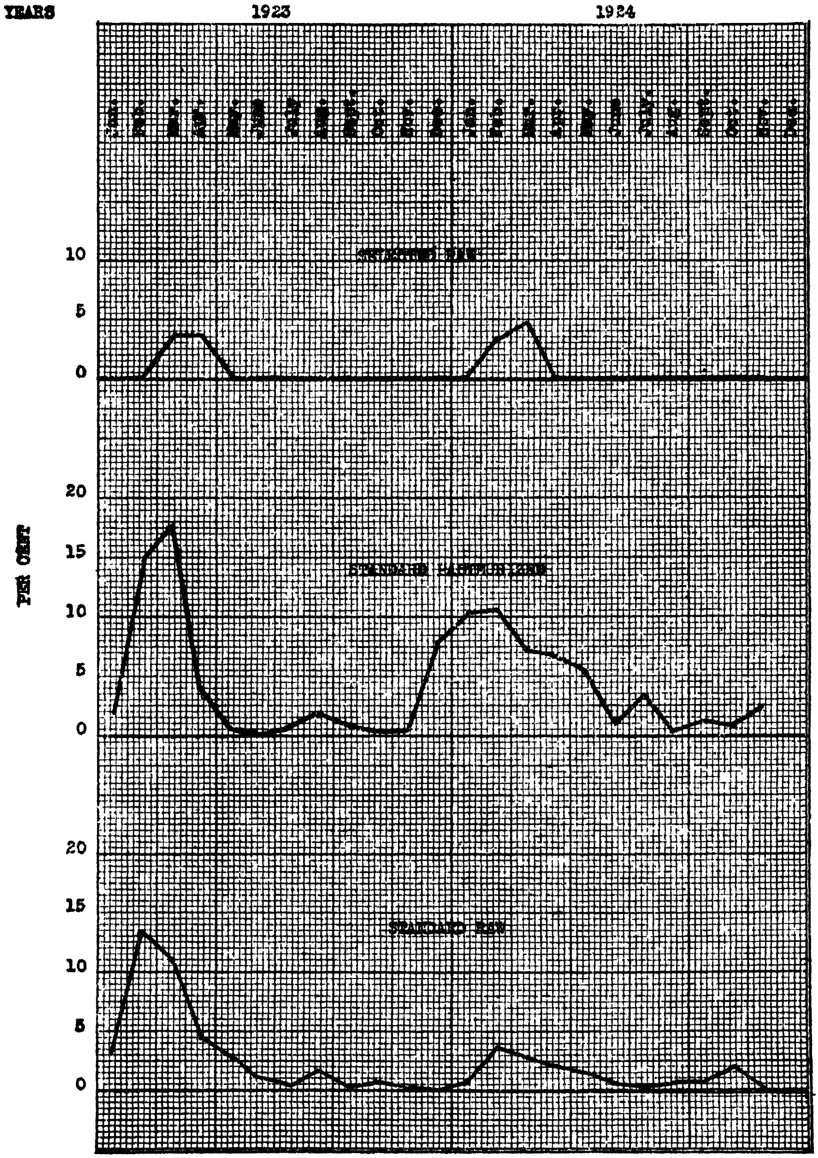

FIG. 1

were incubated for twenty-four hours at 37°C. and then plated on Ayer's milk powder Agar A. In this way pure cultures were secured and were kept growing in milk by transferring every two or three weeks. In all, 52 cultures were secured. Twenty-one of these came from raw milk, 26 from pasteurized milk, and 5 from ice cream. It should be mentioned that the five cultures from ice cream were from plates which were not typical. The colonies were quite small, but they developed in such a manner that approximate check counts could be made from the different dilutions.

The 21 cultures from raw milk included cultures from the raw milk of 5 different pasteurizing plants. The 26 cultures from pasteurized milk included cultures from the pasteurized milk of 7 different pasteurizing plants. The 5 cultures from ice cream included cultures from one plant only.

On morphological examination 50 of these cultures proved to be streptococci, one was a staphylococcus and one a spore former. These last two cultures when replated on plain agar produced large colonies. All of the streptococcus cultures when plated in pure culture on plain agar at pH 6.8 to 7.0 either produced typical small colonies or failed to show up as colonies. Sixteen of the streptococcus cultures were studied further. We have not attempted to identify these cultures because they do not seem to be typical of any well defined type. The characteristics of these organisms are given in table 1.

In so far as we have examined these cultures we have for the most part followed the procedure of Ayers and his collaborators in their studies of the streptococcus from different sources. (Exceptions which should be noted are the use of rabbits' blood and North's medium in our blood agar plates, and of National Aniline and Chemical Co. methylene blue in concentration of 0.1 cc. of 0.5 per cent solution in 10 cc. of milk.) This was done in order that we might determine if possible by comparison with the organisms which they studied the probable source of these "pin point colony" producing streptococci. In this we were disappointed since our cultures do not seem to be typical of any class which they studied, with the possible exception of the 7

TABLE 1

Characteristics of "pin point colony" streptococci

NUMBER OF CULTURES	SOURCE	HEMOLYSIS	CHAINS	METHYLENE BLUE 37°C.	LITMUS MILK	FERMENTATION*							ACID PRODUCTION IN LITMUS MILK†			COLONIES ON PLAIN AGAR WHEN IN PURE CULTURE
						Glucose	Lactose	Sucrose	Salicin	Mannitol	Raffinose	Inulin	20°C.	37°C.	50°C.	
2	Ice cream	Alpha green	Short	Complete reduction 18 hours, coagulated 24 hours	Acid coagulated	+	+	+	+	+	+	−	++	+++	−	+
1	Pasteurized milk	Alpha green	Long	Complete reduction 18 hours, coagulated 24 hours	Acid coagulated	+	+	+	−	−	−	−	−	++	−	−
2	Pasteurized milk	Alpha green	One long; one short	Partial reduction 48 hours, coagulated 72 hours	Acid coagulated	+	+	+	−	−	−	−	−	++	−	-
3	Pasteurized milk	Gamma green	Long		Acid coagulated slowly	+	+	+	−	−	−	−	−	+	−	-
1	Pasteurized milk	Alpha green	Short		Acid	±	±	−	−	−	−	−	±	+	−	-
7	Raw milk	Alpha green	Long		Weak acid	−	−	−	−	−	−	−	±	+	−	+

* Culture tested with methyl red. + indicates final acid color pH 4.6 or lower. ± indicates neutral range approximately pH 5.4 to 4.8. − indicates that cultures did not develop enough acid to appreciably change the color of methyl red pH 5.8 or higher.

† − indicates no change in the color of litmus after five days' incubation. ± indicates the first slight change in three to five days. + indicates a definite change in three days. ++ indicates a definite change in two days. +++ indicates a definite change in one day.

cultures from raw milk, which show some similarity to their cultures of *Streptococcus acidominimus*, which they found was one of the minor udder types.

In order to approximate the optimum growing temperature for these organisms, three sets of litmus milk tubes were inoculated in the same manner. One set was incubated at 20°C., one set at 37°C. and one set at 50°C. The rapidity with which acid was produced as determined by a change in the color of litmus, was taken as indicative of the comparative rate of growth at the different temperatures. The results of these tests are included in the table.

In the last column of the table is indicated the ability of these organisms to produce colonies on plain agar (pH 6.8 to 7.0) when inoculated in pure cultures and without the addition of milk.

DISCUSSION

It would appear from the data presented above that what we have considered "typical pin point colonies" have a fairly definite seasonal occurrence. In each of two years we have found them to occur with the greatest frequency in the months of January, February, March, and April. They occurred at the same time in both raw and pasteurized milk. This fact would indicate that the "pin point colony trouble" which we have been observing has its origin at the farm. However, in examining the organisms responsible for these colonies we found several varieties of streptococci, and those from pasteurized milk were different than those from raw milk. Our cultures were not collected with the idea of determining the predominant types in raw milk and pasteurized milk. To establish a difference in type would require the study of a great many more cultures. Other points to consider are the dilutions of the milk in the original plates and the flora (other than "pin point" colony organisms) present in the milk. What part other organisms may play in making conditions suitable for the growth of a particular organism when they are associated on the same plate is a subject we know little about.

It should be noted that these organisms occur in the milk

supply of Baltimore coincident with the spring freshening season for cows on the Baltimore milk shed. Whether streptococci are more numerous in the udder of cows or about the farm at this time of the year we do not know. However, on purely theoretical grounds, udder trouble would be expected to be quite prevalent at this time of the year.

We do not propose to draw any conclusions from this work as to the connection between these organisms and mastitis in cattle. Much more work would be necessary to determine such a fact. However, it may be said that should such a relationship be established, the occurrence of "typical pin point colonies" when due to streptococci might prove to be a valuable index by means of which udder trouble could be located; just as "pin point colonies" when due to thermophiles have proven of value in locating trouble in pasteurizing plants.

SUMMARY

The literature indicates that "pin point colonies" on milk plates may be due to a variety of causes, such as crowded plates, reaction of medium, thermophilic organisms, and streptococci.

We have found "pin point colonies" to occur on our routine plates for the control of milk with the greatest frequency in the months of January, February, March and April.

The organisms responsible for the colonies were found to be streptococci.

The possible relationship of these organisms to udder trouble and the significance which could be attached to "pin point colonies" when due to streptococci should it be shown that these organisms indicate mastitis have been discussed.

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THE OXALIC ACID TEST FOR INDOL

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The oxalic acid test for indol is one which has received little attention in spite of certain features which would seem to render it of considerable value. It was apparently first applied to bacteriological work by Pittaluga (1908) and Morelli (1909), but failed to gain general recognition and only a few other references to it are to be found. Recently Holman and Gonzales (1923) have again brought forward the method and believe that it is entitled to more widespread use.

The test is based upon the formation of a pink color upon contact, under proper conditions, of volatile indol and dry oxalic acid crystals. Absorbent paper or filter paper is dipped in an aqueous solution of oxalic acid, dried, cut into strips and a strip of the paper suspended from the cotton stopper in the mouth of the tube containing the culture to be tested. As much of the surface of the paper as possible should be exposed inside the tube, care being taken that it does not come in contact with the culture. If indol is formed it volatilizes at incubator temperature, or even at room temperature, and the oxalic acid paper becomes pink. In the absence of indol the paper remains white. Holman and Gonzales report obtaining positive tests on white tape or on absorbent cotton plugs which were dipped in oxalic acid solution and dried.

Although the advantages of such a test have been emphasized before, nevertheless it may be permissible to restate them here, since many are not familiar with the method. In the first place, the color indicative of a positive test is obtained on the paper or cotton plug in the top of the tube, thus automatically eliminating non-volatile compounds related to indol which might give

confusing color reactions when the test reagents are added directly to the medium. The culture is not destroyed by performing the test so that the production of indol, or at least its volatilization from the medium, can be followed through a considerable period on the same culture, inserting new oxalic acid papers after the first have become pink. Also, the test can be applied to cultures grown on solid media such as agar slants or to those grown in very turbid media or in infusions of chopped meat where the usual tests could not be performed. In view of these advantages it would seem that this method is deserving of more attention than it has received in the past. Our purpose has been to review it with especial reference to its delicacy and accuracy as compared with the more commonly used tests.

The factors controlling development of the pink color upon contact of indol and oxalic acid are apparently not entirely understood. The color does not appear if the oxalic acid paper is wet, nor does it appear upon mixture of solutions of indol and oxalic acid. We have found that filter paper strips dipped in a mixture of indol and oxalic acid solutions of varying concentrations remained colorless until the papers had dried, whereupon the pink color appeared. Holman and Gonzales (1923) believe it essential that the crystals of oxalic acid must be very small or distributed on some finely divided material, otherwise the pink color does not appear. They report that there was no color change when indol was allowed to volatilize through packed oxalic acid crystals.

Before comparing this method with other indol tests it seemed desirable to conduct a few preliminary experiments with regard to preparation of the oxalic acid papers. Attention was given especially to the strength of the oxalic acid solution and to the type of paper used. Solutions containing respectively 2, 4, 6, 8, 10 and 12 grams of oxalic acid, $(COOH)_2 \cdot 2H_2O$, per 100 cc. of distilled water were prepared and filter papers immersed in each of them. The papers were then air dried, cut into strips and used for comparative tests with sterile broth in tubes to which small amounts of a weak solution of indol had been added. In these tests the papers prepared from the two weakest solutions

exhibited a lighter shade of pink, especially with small amounts of indol, and seemed to be less desirable. Little if any difference could be detected between the others. In a later experiment a hot saturated solution of oxalic acid was used for impregnating the filter paper. The results secured here appeared to offer no advantage in depth of color. Several different brands of filter paper, both hard and soft, and a special bibulous paper were used with approximately the same results. Absorbent cotton plugs moistened in the oxalic acid solution and dried were also used. It seemed easier, however, to prepare the papers beforehand and to use them as needed rather than to treat each cotton plug separately, and so throughout our work we have used filter papers or bibulous paper impregnated in an 8 to 10 per cent solution of oxalic acid.

The effect of the reaction of the medium upon the volatility of indol would seem to be important since it might affect the delicacy of the test. Zoller (1920) has reported the effect of reaction upon the volatilization of indol from a medium containing 2 per cent Difco pepton, 0.1 per cent glucose and 1 per cent dibasic potassium phosphate adjusted to varying hydrogen ion concentrations. Distillation was accomplished over the free flame of a Bunsen burner. At pH 5.0, 81 per cent of the indol distilled in the first 75 cc.; at pH 7.0, 92.5 per cent; at pH 8.0 to 10.5, 99 to 100 per cent of the indol appeared. In another experiment, air bubbled for one hour through an aqueous indol solution held at 50°C. removed slightly less than 20 per cent of the indol at pH 5.0, about 31 per cent at pH 6.0, 38 per cent at pH 7.0 and 45 to 48 per cent at pH 8.0 to pH 10.0. While the greatest lost in each case occurred in the range of pH 8.0 to 10.5. it is nevertheless apparent that a fairly large proportion escaped even at the more acid reactions. Whether sufficient indol to give a positive test with oxalic acid paper would escape from an acid medium at incubator temperature remained to be determined.

A sample of plain nutrient broth (1 per cent Difco pepton and 0.3 per cent meat extract) was divided into four separate lots and each was adjusted to a different degree of acidity or alkalinity.

Varying quantities of a dilute indol solution were then added to each of the different lots to give concentrations ranging from 0.001 to 0.2 mgm. indol per cubic centimeter of broth. The broth was tubed in 5 cc. quantities and oxalic acid papers inserted in the tubes. This experiment was repeated several times with samples ranging from pH 4.8 to 9.2. In each case a distinct positive test was obtained within twenty-four hours in the tubes containing the greatest concentrations of indol, irrespective of reaction. Where only small quantities of indol were present the effect of the reaction was questionable. In one experiment with 0.005 mgm. indol per cubic centimeter of broth, a very faint test was obtained after forty-eight hours in two tubes, at pH 8.0 and 9.2 respectively; two other tubes, at 4.8 and 6.8, gave questionable or negative results. In several other experiments the distinction was not as clear cut and very little if any difference was apparent between acid and alkaline media. However, in a general way it may be stated that the test appeared to be reliable at different reactions in all cases except those where only small amounts of indol were present. These cases gave conflicting results. Evidently there is at least a possibility that cultures forming only small quantities of indol or those grown in a medium of low tryptophane content, especially if acid or neutral in reaction, may be erroneously recorded as indol negative.

A comparison of the delicacy of the oxalic acid test with several of the commonly used indol tests was next made. For this purpose the following were employed: The Ehrlich test with paradimethylamidobenzaldehyde, the Salkowski test with sodium nitrite and sulphuric acid, the vanillin test and of the Goré test. The technic used in applying the first three of these tests followed that recommended in the Manual of Methods by the Committee on Bacteriological Technic. In each case the reagents are added directly to the culture. The Goré test (1921) has recently been described and is essentially a modification of the Ehrlich test. The reagent with the addition of potassium persulphate is applied to the cotton stopper instead of being added to the medium, the culture is heated to drive off indol and the typical color of the positive test appears on the cotton.

These tests were compared by applying them to dilute solutions of indol in broth or to cultures. A 0.1 per cent solution of indol was made in 95 per cent ethyl alcohol and from this further dilutions were made in sterile broth to give a series of tubes with concentrations of indol ranging from 0.1 to 0.0001 mgm. per cubic centimeter of broth. The different lots of broths with varying concentrations of indol were then filled into test tubes in 5 cc. amounts and each of the tests applied to a series of tubes. The results of the oxalic acid test were recorded after incubation at 37°C. In another experiment a broth culture of *Bact. aerogenes* (indol negative) was used for dilution of the indol instead of the sterile medium. A forty-eight-hour culture, exhibiting luxuriant growth and heavy turbidity, was killed by steaming in the Arnold and varying quantities of indol were then added to the killed culture and the tests applied as before. In this case, of course, the colors obtained in those tests where the reagents are added directly to the medium must be observed through the turbid growth and the conditions more nearly simulate those under which the tests are usually applied in bacteriological work.

The result of these experiments, shown in tables 1 and 2, are essentially similar in that the Ehrlich, Vanillin and Goré tests are more sensitive than the oxalic acid paper. When the tests are applied to clear broth (table 1) the Ehrlich and vanillin tests were capable of detecting indol in quantities as small as 0.001 mgm. per cubic centimeter of broth, i.e., 1 part per million. However, when applied to turbid cultures the highest dilution to give a faintly positive test was that containing 0.002 mgm. indol per cubic centimeter, or 1 part in 500,000. Also, under these conditions the Goré test was as sensitive as the others. The oxalic acid papers are apparently less delicate, since a positive test was never obtained with less than 0.005 mgm. indol per cubic centimeter, or 1 part in 200,000. It should be pointed out in this connection that the reaction of the broth as well as of the killed culture to which these tests were applied was pH 6.8. In our hands the Salkowski test has given the least satisfactory results of any of the tests studied.

Following our work with indol added to sterile broth or killed cultures, we next used cultures grown in several different media:

TABLE 1

Results of the various tests applied to known dilutions of indol in sterile broth

		Dilutions of indol per cubic centimeter of broth							Control
		0.1 mgm.	0.01 mgm.	0.005 mgm.	0.002 mgm.	0.001 mgm.	0.0005 mgm.	0.0002 mgm.	
Oxalic acid paper†	24 hours	+	?	–	–	–	–	–	–
	48 hours	++	+	?	–	–	–	–	–
	96 hours	+++	+	+	–?	–	–	–	–
Goré		+++	++	++	+	–	–	–	–
Ehrlich		+++	+++	++	+	+	–*	–*	–*
Vanillin		+++	+++	++	+	+?	–	–	–
Salkowski		+	+	?	–	–	–	–	–

Negative, weak, fair, and strong tests are indicated, by –, +, ++, or +++, respectively.

* Confusing colors appeared in these tubes.

† Oxalic acid tests were held at 37°C. Incubation for a period longer than four days failed to bring out positive tests in any greater dilutions.

TABLE 2

Results of the various tests applied to known dilutions of indol in a killed broth culture

		Dilutions of indol per cubic centimeter of broth culture							Control
		0.1 mgm.	0 01 mgm.	0.005 mgm.	0.002 mgm.	0.001 mgm.	0.0005 mgm.	0.0002 mgm.	
Oxalic acid paper†	24 hours	+	?	–	–	–	–	–	–
	48 hours	++	+	?	–	–	–	–	–
	96 hours	+++	++	+	–	–	–	–	–
Goré		+++	++	+	+	–	–	–	–
Ehrlich		+++	+++	++	+	–?*	–*	–*	–*
Vanillin		+++	+++	++	+	?	–	–	–
Salkowski		+	+	–?	–	–	–	–	–

Negative, weak, fair, and strong tests are indicated by –, +, ++, or +++, respectively.

* Confusing colors appeared in these tubes.

† Oxalic acid tests were held at 37°C. Incubation for a period longer than four days failed to bring out positive tests in any greater dilutions.

(1) 2 per cent solution of Difco pepton; (2) 2 per cent Witte pepton; (3) 2 per cent solution of aminoids (50 per cent of total nitrogen as amino nitrogen) and, (4) a casein digest as prepared

by Kulp and Rettger (1924). In a general way the results here corroborated those previously obtained in that the oxalic acid

TABLE 3

Results of various indol tests in different media

METHOD OF TESTING	MEDIUM USED	BACT. COLI		CONTROLS	
		Strain 1	Strain 2	*Bact. cloacae*	Sterile medium
Oxalic acid	Difco pepton, 2 per cent	0	+	0	0
	Witte pepton, 2 per cent	+?	+	0	0
	Aminoids, 2 per cent	0	+	0	0
	Casein digest	+	++	0	0
Goré	Difco pepton, 2 per cent	++	+++	0	0
	Witte pepton, 2 per cent	++	+++	0	0
	Aminoids, 2 per cent	+	+++	0	0
	Casein digest	+++	+++	0	0
Ehrlich	Difco pepton, 2 per cent	+	+++	0*	0*
	Witte pepton, 2 per cent	+++	+++	0*	0*
	Aminoids, 2 per cent	+	+++	0*	0*
	Casein digest	+++	+++	0*	0*
Vanillin	Difco pepton, 2 per cent	++	+++	0†	0†
	Witte pepton, 2 per cent	++	+++	0†	0†
	Aminoids, 2 per cent	++	+++	0	0
	Casein digest	++	+++	0	0
Salkowski	Difco pepton, 2 per cent	++	++	0	0
	Witte pepton, 2 per cent	++	++	0	0
	Aminoids, 2 per cent	0	++	0	0
	Casein digest	+	++	0	0

In this series of tests all tubes were incubated at 37°C. for 48 hours.

Negative, weak, fair and strong tests are indicated by 0, +, ++, and +++, respectively.

* Upon use of the Ehrlich reagent, confusing colors appeared in the uninoculated medium and in the indol-negative cultures. These were usually a lavender and could be distinguished from the reddish purple or deep rose tint produced by contact with indol. The same lavender shade appeared along with the positive tests in many of the indol-positive cultures.

† Confusing colors, other than that of the positive test, appeared frequently in these cases.

test is apparently not as delicate as several of the others, for occasionally a weakly positive culture gave negative results,

particularly when grown in a medium poor in tryptophane. This point is illustrated in table 3 which is representative of other similar experiments. Here *Bact. coli* 1 usually gave a weaker test, by any of the methods, than did no. 2 which was a vigorous indol producer. This is especially noticeable with the Difco pepton and aminoid solutions and it is here that the oxalic acid test gave negative results upon several occasions. On the other hand, the oxalic acid test is apparently reliable in that false positive results were never secured when applied to a rather long list of indol negative cultures and to tubes of sterile media. in addition to those shown in the table. We have also applied the oxalic acid test and the Goré test to cultures grown on agar slants with results essentially similar to those secured by the use of liquid media.

When using the oxalic acid papers with indol-producing cultures it was noticed that the pink color tended to fade after several days if the papers were left in the tube. The loss of color first appeared at the bottom and edges of the paper and gradually spread. This same phenomenon was mentioned by Holman and Gonzales (1923) who suggested that it might be due to the influence of ammonia. It would seem to be caused by some volatile product of bacterial metabolism since in our work it was not observed in the tubes of sterile medium to which indol had been added. In such tubes the pink color gradually changed to an orange brown tint upon long standing, but did not fade. The fading does not seriously interfere with reading of the tests.

The above results may be compared with those obtained by several other investigators. Pittaluga (1909) states that the oxalic acid test gave a positive result after three days with a 1 to 200,000 dilution of indol and that the color even appeared at times in dilutions as high as 1 part per million. Zipfel (1912) found the Ehrlich-Böhme test to give positive results in dilutions as high as 1 to 4,000,000 in aqueous solutions of indol, but in a pepton solution no test was recorded higher than 1 part per million. The Salkowski test was sensitive to 1 part per million in aqueous solution and to 1 part in 400,000 in broth. He also employed oxalic acid paper and although he does not state the

delicacy of this test, he regards it as a simple and valuable control to other tests. Malone and Goré (1921) compared the various methods of applying the Salkowski and the Ehrlich tests but did not use the oxalic acid test. With certain modifications of the Ehrlich test and with the Goré test, they obtained positive results in somewhat higher dilutions of indol than we have been able to do in the present study. They found the nitrosoindol reaction, or Salkowski test, to be very unsatisfactory as ordinarily performed, a conclusion with which we are in complete agreement. Fellers and Clough (1925) have recently published an extensive review of the various methods, in which they found the Ehrlich reagent to be the most delicate and satisfactory. The Goré modification was not employed in their work. The oxalic acid test gave less satisfactory results than several of the more commonly used methods. To increase the delicacy and reliability of the Ehrlich reagent they suggest distillation of the culture, followed by extraction of the distillate and application of the test reagent. By this procedure quantitative results may be secured and they state it is accurate to 1 part in 25,000,000.

In comparing the merits of the oxalic acid test with those of the other procedures employed in our work it is evident that the test is not as delicate as several of the others, notably those which make use of the Ehrlich reagent or vanillin. However, the specificity of both the Ehrlich reagent and vanillin has been questioned and if added directly to the culture medium they may react with phenolic and other compounds in addition to indol. Throughout our work the oxalic acid papers have never shown confusing colors or false positive tests and, in addition, they may be used for detecting indol production on solid media. Although these advantages are somewhat offset by the lack of sensitiveness, it should be noted that in preparing cultures for indol tests the oxalic acid papers may be employed without destruction of the culture so that any other method may then be applied to the same tube.

Of the several indol tests employed in our work, we are inclined to favor the Goré method as the most satisfactory which

may be used where large numbers of cultures are to be examined. It possesses most of the advantages of the oxalic acid method and in addition it is more delicate and the color of the positive test is deeper and more striking than the pink tint of the oxalic acid papers. In our hands the Goré test has never yielded the confusing colors or false positive tests that are secured when the reagents are added directly to the culture, and at the same time appears to be as delicate under these conditions. Also, it has given pronounced and striking positive tests when applied to agar slant cultures of indol-producing organisms, even when a pepton of low tryptophane content was used in the medium.

The method of testing for indol recently recommended by Fellers and Clough (1925) evidently is more delicate than any of the foregoing since they state that it is accurate to 1 part in 25,000,000. While such a method is undoubtedly very useful in detecting and estimating small quantities of indol, the length of the procedure (distillation, ether extraction of the distillate, washing the ether extract and subsequent evaporation of ether) would seem to constitute a decided disadvantage when studying a large number of cultures. It is here that the Goré method would appear to find its greatest usefulness since it may be applied readily to large numbers of cultures, whether grown in liquid or solid media. It has also been found very satisfactory in connection with the study of different bacterial types in classroom work.

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THE BLOOD-AGAR PLATE FOR SPORE-FORMING ANAËROBES

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The advantages of the blood-agar plate for the isolation and grouping of streptococci are well known. It now seems that the blood-agar plate may be equally useful in isolating and grouping another genus of bacteria, the spore-forming anaërobes. Under suitable conditions of anaërobiosis this medium has proved very satisfactory for securing growths, and the effect of these bacteria on hemoglobin resembles that obtained with streptococci, that is, some spore-forming anaërobes are hemolytic; some produce methemoglobin, and others have no effect on the red blood cells. It appears also that the plate method is more suitable for quickly securing pure cultures than the deep tube methods, so much used in the past, principally because the gas formed by many of these bacteria escapes from the thin layer of agar more readily than from the deep layer and thereby prevents mixing of the growths. Under similar physical conditions the anaërobic spore-formers should be as easy to secure in pure culture as, for example, the streptococci, although the literature gives the impression that pure cultures may be obtained only after long and tedious effort. More recently the Barber single cell method has been adopted by certain bacteriologists as the only reliable means of separating the anaërobes.

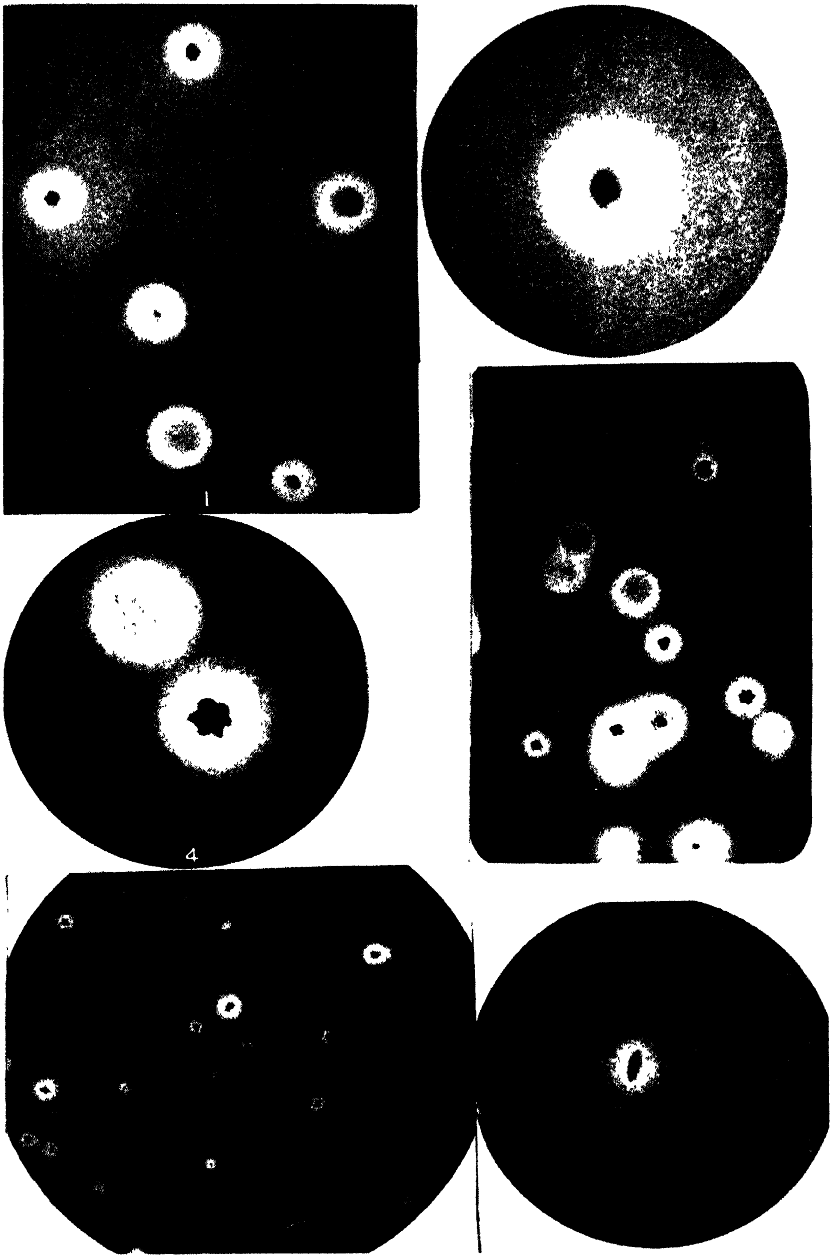
During the last year the blood-agar plate has been used as a routine for securing pure cultures of anaërobes at postmortem examinations with good results. I shall now present more particularly a tentative classification of pure cultures, obtained from

other sources, according to their effect on the blood-agar plate. Zeissler, used blood-agar-streak plates for growing *Clostridium welchii* and *Clostridium oedematis-maligni* and reports very characteristic surface colonies, stating that this method is most satisfactory for proving the purity of cultures. Richardson and Dozier speak of isolating delicate anaërobes on blood-agar plates, anaërobic conditions being obtained by the method of McIntosh and Fildes. They give no details as to cultures, but later Wagner, Dozier, and Meyer, using the same anaërobic method, describe the growth of *Clostridium botulinum, Clostridium sporogenes* and *Clostridium tetani* on poured blood-agar plates. Wheeler and Humphreys used blood-agar plates for growing anaërobes, securing anaërobic conditions in a jar exhausted with hydrogen. They were able to secure characteristic colonies of *Clostridium welchii, Clostridium botulinum, Clostridium sporogenes,* and *Clostridium histolyticum,* but found that *Clostridium tetani* and *Clostridium putrificum* grew poorly. Wagner reports that Pfenninger was unable to grow *Clostridium botulinum type C,* and the parabotulinus organism of Seddon on blood-agar plates. Hall has made extensive use of blood-agar slants for the cultivation of anaërobes, securing anaërobic conditions by Wright's method.

EXPERIMENTAL DATA

The medium used was hormone agar made after Huntoon's formula, with 1.5 per cent agar, and sterilized in flasks in amounts of 50 and 100 cc. The agar was melted and cooled to 45°C.

FIG. 1. CLOSTRIDIUM WELCHII (No. 2 HALL)
Double method, twenty-four hour culture. × 2
FIG. 2. CLOSTRIDIUM WELCHII (No. 2 HALL)
Double method, twenty-four hour culture. × 6
FIG. 3. CLOSTRIDIUM CENTROSPOROGENES (No. 76 HALL)
Double method, forty-eight hour culture. × 2
FIG. 4. CLOSTRIDIUM CENTROSPOROGENES (No. 76 HALL)
Double method, forty-eight hour culture. × 6
FIG. 5. CLOSTRIDIUM OEDEMATIS-MALIGNI (No. 18 HALL)
Double method, twenty-four hour culture. × 2
FIG. 6. CLOSTRIDIUM OEDEMATIS-MALIGNI (No. 18 HALL)
Double method, twenty-four hour culture. × 6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and 5 per cent human blood added; it was then dispensed into sterile tubes, 10 to 12 cc. to each tube. Serial inoculations were made from the material to be cultured, and plates poured. Anaërobic conditions were secured by means of a Novy jar exhausted with hydrogen, or by combining this with the alkaline pyrogallol method. In filling the jar with hydrogen the apparatus was so arranged that hydrogen entered the top of the jar while the air was drawn off from the bottom by means of a rubber tube attached to the glass tube in the stopper and long enough to reach to the bottom of the jar when the cover was in place. The ground-glass seals between the two halves of the jar and around the stopper were kept well coated with vaselin, and the jar, when in use, was held together with small clamps. A hydrogen cylinder fitted with a reducing valve was found to be the most convenient source of hydrogen. The hydrogen was allowed to run into the jar and the gas issuing from it was led into a pan of water by a tube with an inside diameter of between 4 and 5 mm. When the bubbles appeared at the rate of about 175 a minute it was assumed that the air could be largely removed from the jar in from twenty to thirty minutes, and if a tube of the gas was caught over water after this interval it would burn with very little explosion when ignited.

By this method the anaërobes which can tolerate small amounts of oxygen will grow well, but others grow poorly or only on very heavy inoculation. Repeating the process of exhaustion after half an hour to remove traces of oxygen which might diffuse out from the plates gave no better results. Therefore the remaining oxygen was absorbed in alkaline pyrogallate, which was done by placing an empty plate in the bottom of the jar to keep the others out of the liquid, and adding about 15 to 20 gm. of pyrogallic acid for a jar of approximately 3 liters capacity. It was necessary to have a jar whose diameter was somewhat larger than that of the plates. After exhausting with hydrogen about 50 cc. of 5 per cent sodium hydroxid was aspirated back into the bottom of the jar through the delivery tube, care being taken to turn the stopcock in the jar before air entered. It was found that cultures which grew poorly with the first method grew freely on the addi-

tion of the small amount of alkaline pyrogallate; it was therefore concluded that these cultures were inhibited by small amounts of oxygen. In some cases twenty-four hours is sufficient to secure well developed colonies, but for the majority forty-eight hours is preferable.

While the list of anaërobes studied by this method is not complete, enough of the more common ones have been examined to give a fairly good idea of the groups that may be expected. The difficulties attendant on securing authentic cultures for comparison make a complete study of all the described anaërobes a time-consuming project. Several platings were done on each culture, each being tried by both of the methods of anaërobiosis described. The following species have been studied, the numeral following each name indicating the number of strains: *Clostridium welchii* (10); *Clostridium centrosporogenes* (1); *Clostridium oedematis-maligni* (vibrion septique) (2); *Clostridium chauvei* (1); *Clostridium bifermentans* (2); *Clostridium histolyticum* (1); *Clostridium novyi* (1); *Clostridium tetani* (2); *Clostridium botulinum Type A* (3); *Clostridium botulinum Type B* (3); *Clostridium sporogenes* (1); *Clostridium tertium* (3) and *Clostridium putrificum* (1). The deep colonies were preferable to surface colonies for differentiation, since surface colonies are so largely influenced by moisture, and since in certain cases surface colonies are very few as compared with deep colonies. The following grouping is proposed, to serve, first, as a rapid laboratory method of indicating the more important pathogens which belong to the hemolytic group, and, second, as a guide to their more detailed identification.

BLOOD-AGAR GROUPING OF SPORULATING ANAËROBES

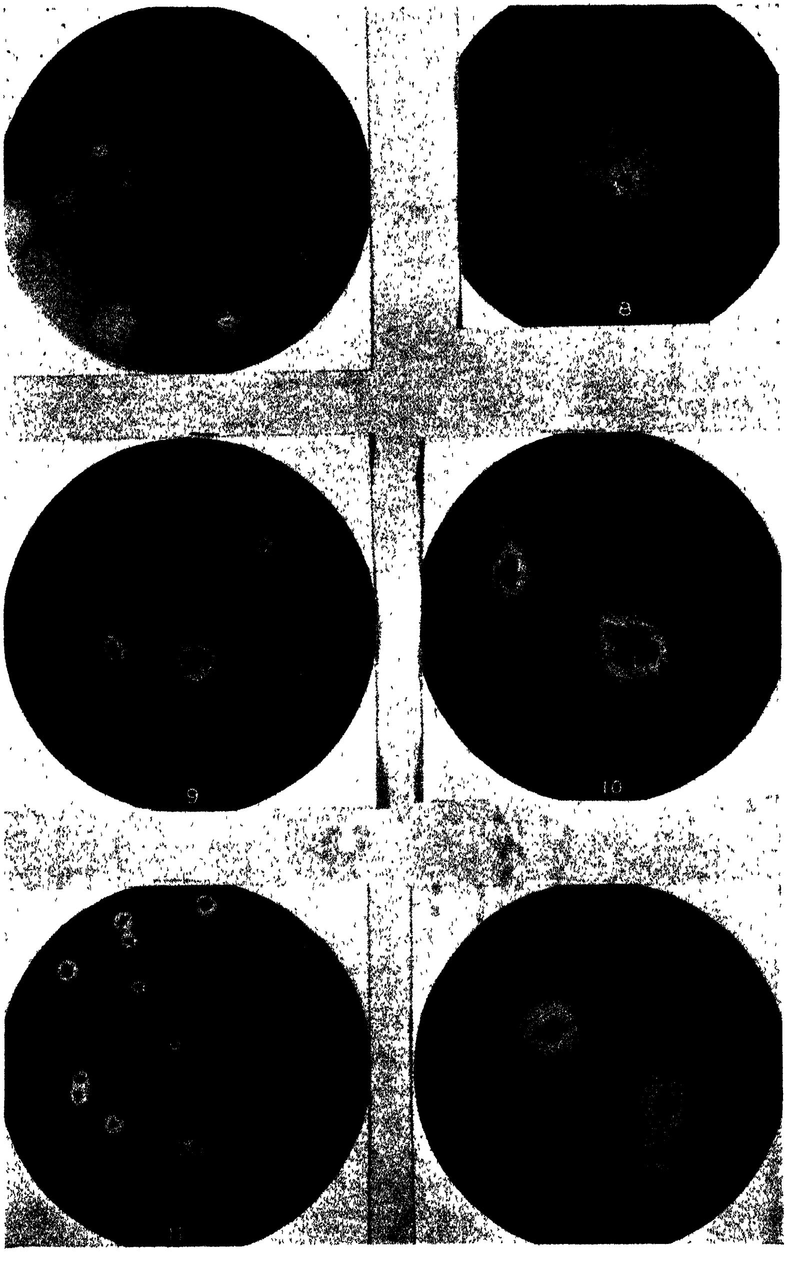
Group I. Hemolytic

- A. Grow well in anaërobic jar exhausted with hydrogen (single method)[2]
  - 1. Zone of hemolysis large compared to deep colony
    - a. Deep colony large, compact, often irregular, hemolysis clear

[2] For convenience the method of exhaustion with hydrogen is called the single method. When alkaline pyrogallate is also used it is called the double method.

(1) Zone of hemolysis surrounded by a deeper red zone
Clostridium welchii
(2) Zone of hemolysis not surrounded by a deeper red zone
Clostridium centrosporogenes
b. Deep colony small, compact, often lens-shaped
Clostridium oedematis-maligni (vibrion septique)
Clostridium chauvei
2. Zone of hemolysis small compared to size of deep colony
a. Deep colony filamentous on margin
Clostridium bifermentans
b. Deep colony compact and irregular
Clostridium histolyticum
B. Grow well only under strict anaërobic conditions (double method)
1. Zone of hemolysis large compared to size of deep colony
a. Deep colony compact, irregular, hemolysis clear
Clostridium novyi
b. Deep colony, filamentous on margin
Clostridium tetani
2. Zone of hemolysis small compared to size of deep colony
a. Deep colony compact, disc-shaped or irregular
Clostridium botulinum
b. Deep colony filamentous on margin
Clostridium sporogenes
Group II. Not hemolytic, producing green zone around colonies
A. Grow well in anaërobic jar exhausted with hydrogen
1. Deep colony small, compact, lens-shaped
Clostridium tertium

FIG. 7. CLOSTRIDIUM CHAUVEI (NO. 6 HALL)
Double method, forty-eight hour culture. × 2
FIG. 8. CLOSTRIDIUM CHAUVEI (NO. 6 HALL)
Double method, forty-eight hour culture. × 6
FIG. 9. CLOSTRIDIUM BIFERMENTANS (NO. 102 HALL)
Double method, forty-eight hour culture. × 2
FIG. 10. CLOSTRIDIUM BIFERMENTANS (NO. 102 HALL)
Double method, forty-eight hour culture. × 6
FIG. 11. CLOSTRIDIUM HISTOLYTICUM (NO. 290 HALL)
Double method, forty-eight hour culture. × 2
FIG. 12. CLOSTRIDIUM HISTOLYTICUM (NO. 290 HALL)
Double method, forty-eight hour culture. × 6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Group III. Without effect on red blood cells
  A. Grow only under strict anaërobic conditions
    1. Deep colony punctiform
      Clostridium putrificum

DESCRIPTION OF CULTURES STUDIED

Clostridium welchii

Culture 2. (I. C. Hall) Twenty-four hours' incubation
  Good growth in twenty-four hours or less by both single and double methods
  Surface colony: 2 to 3 mm., round, entire, convex
  Deep colony: 1 to 1.5 mm., lens-shaped or irregularly lobate dense masses, with no filaments on margin (× 100)
  Hemolysis: Marked. A primary clear zone surrounded by a wide diffuse zone, which is in turn bordered by a zone of darker red than the surrounding medium (figs. 1 and 2)
  Morphology: Large rods of varying lengths, Gram-positive and Gram-negative. Rods often appear granular

Clostridium centrosporogenes

Culture 76. (I. C. Hall) Forty-eight hours' incubation
  Grow well by both single and double methods, forty-eight hours required for well developed colonies
  Surface colony: 1 to 2.5 mm., round, entire, or slightly irregular, some with raised centers, some with depressed centers
  Deep colony: Dense irregular multilobulate masses, no filaments on margin (× 100)
  Hemolysis: Clear zone whose diameter is 2 to 2.5 times that of the deep colony (figs. 3 and 4)
  Morphology: Large rods, much like *Clostridium welchii* except larger

CLOSTRIDIUM OEDEMATIS-MALIGNI (VIBRION SEPTIQUE)

Culture 18. (I. C. Hall) Twenty-four hours' incubation
  Grow well by both single and double methods
  Surface colony: Variable in size and shape, some flat spreading films covering a quarter of the plate; some small (1 mm.) with root-like processes at margin, some small (0.5 mm.) round, entire, convex

Deep colony: Punctiform to 1 mm., disc-shaped or irregular, with knobs or fan-like projections, no filaments on margin (× 100)

Hemolysis: Some colonies show a small clear zone slightly larger than the colony itself, some a large diffuse zone three to five times the diameter of the colony (figs. 5 and 6)

Morphology: Some long rods, others medium or short, Gram-positive but not strongly so. Bipolar staining, and lightly staining barrel-shaped forms with or without polar granules common

Culture 421. Isolated September 11, 1924, at postmortem from a case of peritonitis. Cultural characters, morphology and pathogenicity for rabbits on intramuscular inoculations indicated *Clostridium oedematis-maligni*

On blood-agar plates it behaved in all respects like the preceding culture

Clostridium chauvei

Culture 6. (I. C. Hall) Forty-eight hours' incubation

Good growth by both single and double methods

Surface colony: 1 to 1.5 mm., round, entire, convex, slightly opaque

Deep colony: 0.2 mm., lens-shaped, compact, no filaments on margin (× 100)

Hemolysis: A wide zone compared to size of colony (2 to 3 mm.) Many red cells still intact giving a diffuse hazy appearance to the zone (figs. 7 and 8)

Morphology: Medium sized Gram-positive rods, irregular staining, swollen, round or clubbed forms common

Clostridium bifermentans

Culture 102. (I. C. Hall) Forty-eight hours' incubation

Good growth by both single and double methods

Surface colony: Some of small dew drop type, and others like a mat of filaments, rather dry looking

Deep colony: 0.25 to 1 mm., often showing a dense core set in a net work of filaments which contains coarse granules (× 100)

Hemolysis: The zone is not clear cut and extends but little beyond the line of growth (figs. 9 and 10)

Morphology: Large rods, Gram-positive and some partly decolorized. Granular forms common as in *Clostridium welchii*

Culture 507. Isolated at postmortem November 1, 1924, from the blood and spleen of a case of carcinoma of the ovary. The

results on coagulated egg, gelatin, brain broth, blood serum, litmus milk, and sugar broths indicate *Clostridium bifermentans.* This culture is nonpathogenic for rabbits when given intravenously or intramuscularly. On blood-agar plates it grows like culture 102, producing similar deep colonies and the same type of hemolysis.

Clostridium histolyticum

Culture 290. (I. C. Hall) Forty-eight hours' incubation
- Good growth by both single and double methods
- Surface colony: Some 1 mm., round, entire, convex, white (resembling staphylococcus colony), others 3 mm. to 1 cm., flat, spreading, with finger-like projections
- Deep colony: 0.5 mm., compact, lens-shaped, heart-shaped, or irregular, with clear-cut margins having no projecting filaments (× 100)
- Hemolysis: Zone clear and sharply defined, its diameter being about twice that of the colony (figs. 11 and 12)
- Morphology: Medium sized Gram-positive rods, some appear granular

Clostridium novyi

Culture 140. (I. C. Hall) Forty-eight hours' incubation
- Growth poor by single method, but rapid by double method
- Surface colony: 3 to 4 mm., round, flat, with raised center, margin torn and irregular, color yellowish
- Deep colony: Dense irregular masses 0.5 to 1.0 mm. with few short projecting hair-like filaments (× 100)

FIG. 13. CLOSTRIDIUM NOVYI (No. 140 HALL)
Double method, forty-eight hour culture. × 2

FIG. 14. CLOSTRIDIUM NOVYI (No. 140 HALL)
Double method, forty-eight hour culture. × 6

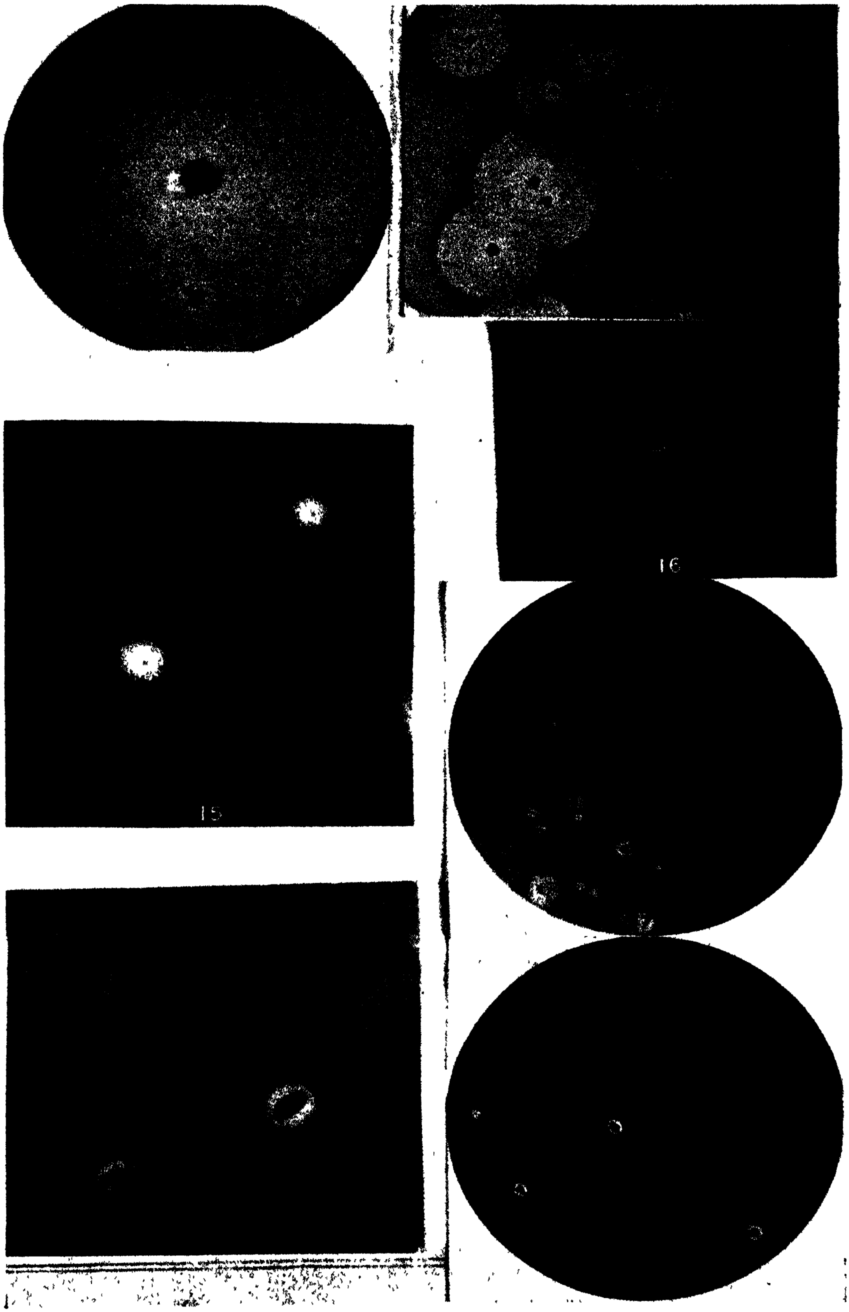
FIG. 15. CLOSTRIDIUM TETANI (No. 1 HALL)
Double method, forty-eight hour culture. × 2

FIG. 16. CLOSTRIDIUM TETANI (No. 1 HALL)
Double method, forty-eight hour culture. × 6

FIG. 17. CLOSTRIDIUM BOTULINUM TYPE A (No. 9 PARK)
Double method, forty-eight hour culture. × 2

FIG. 18. CLOSTRIDIUM BOTULINUM TYPE A (No. 9 PARK)
Double method, forty-eight hour culture. × 6

FIG. 19. CLOSTRIDIUM BOTULINUM TYPE B (No. 178 HALL)
Double method, forty-eight hour culture. × 2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Hemolysis: Zones very large and clear, eight to ten times greater in diameter than the deep colony. There is often a darker red ring just surrounding the hemolytic zone (figs. 13 and 14)

Morphology: Large rods, some curved. Occasional shadow forms, or swollen forms with bipolar staining. Gram-positive

Clostridium tetani

Culture 1. (I. C. Hall) Forty-eight hours' incubation

Growth poor by single method, sometimes a few colonies develop after very heavy inoculation. Growth very rapid by double method

Surface colony: Flat, dry, irregular growths composed of matted filaments

Deep colony: 0.25 to 1 mm. Larger colonies very filamentous (× 100) with large granules among the filaments

Hemolysis: Zone large (eight to ten times greater in diameter than the colony), and hazy, without a clear-cut margin (figs. 15 and 16)

Morphology: Large rods and filaments, Gram-positive

Culture Research. (W. H. Park) Forty-eight hours' incubation

On blood-agar plates this culture grows and appears in all respects like culture 1

Clostridium botulinum Type A

Culture 9 Orr. (W. H. Park) Forty-eight hours' incubation

Growth occurs by double method only

Surface colony: Some are spreading, leaflike or rhizoid, others circular, entire, convex

Deep colony: Some heavy disc-shaped with entire margins 0.5 to 1 mm. Some small to punctiform, irregular, dense masses with or without a few filaments from margin (× 100). The colony shapes of all cultures of *Clostridium botulinum* are more variable than those of any other species studied (figs. 17 and 18)

Hemolysis: The larger disc-shaped colonies have a relatively narrow zone of hemolysis compared to the size of the colony, while the small colonies have a comparatively wide zone. The hemolysis is clear and the zone has a definite margin

Culture Memphis (W. G. McCoy) Forty-eight hours' incubation

Like the preceding culture it produces both spreading and convex

surface colonies, and also disc-shaped and punctiform deep colonies

Culture 372. (I. C. Hall) Forty-eight hours' incubation

The same varieties of deep colony as previously described, also certain ones resembling *Clostridium bifermentans.* The hemolysis is similar in all, that is, with relatively narrow clear zones

Clostridium botulinum Type B

Culture Dixon (W. H. Park) Forty-eight hours' incubation

Culture Nevin (W. G. McCoy) Forty-eight hours' incubation

Culture 178 (I. C. Hall) Forty-eight hours' incubation (See figures 19 and 20 of Culture 178)

What has been said of the type A cultures applies equally well to the type B cultures. The principal types of colony found in all botulinus cultures may be summarized as follows:

Surface: (1) spreading and (2) round, convex colonies

Deep: (1) disc, or lens-shaped colonies with or without coarse projections (fig. 20), and (2) punctiform or small irregular colonies

It was thought at first that these different varieties might indicate a mixed culture; consequently the different types were carefully picked to start new cultures. When these cultures were plated, the same variations occurred as in the original culture. As a further check these cultures obtained by picking different well isolated colonies were tested for toxicity. In all, nine subcultures representing the different colony types to be found in two type A and two type B strains were proved to be toxic for guinea pigs on feeding, and no nontoxic strains were found. This seems to indicate that *Clostridium botulinum* is quite variable as to colony form. The hemolysis, however, is a constant factor.

Clostridium sporogenes

Culture 90. (I. C. Hall) Forty-eight hours' incubation

Growth poor by the single method, but abundant by the double method

Surface colony: 0.25 to 1 cm., fern-like or arborescent, some flat, lobate or ameboid

Deep colony: A dense core 0.2 to 0.5 mm., round or slightly irregular, surrounded by a radiating network of tangled filaments (× 100)

Hemolysis: A clear sharply defined zone 1.5 to 2 mm. in diameter (figs. 21 and 22)

Morphology: Medium-sized rods, Gram-positive

Clostridium tertium

Culture 518. (I. C. Hall) Twenty-four hours' incubation

Growth occurs by either single or double method, but seems to be better if anaërobiosis is not too complete. Incubation for a longer period than twenty-four hours is unnecessary in the cultures studied

Surface colony: Most are round, 0.5 to 1 mm. convex, entire, and slightly milky in color. Some are flat, spreading, 0.5 to 2 cm. with irregular lobate margin

Deep colony: Oval, disc-like, compact bodies, 0.5 mm. without filaments on the margin (× 100)

Hemolysis: Negative. If plates are incubated by the single method there is a narrow faint green zone surrounding the colonies, more marked after the plates have been removed from the jar for an hour or two and the blood has regained its bright red color. *Clostridium tertium* is a representative of the group of anaërobes producing green zones on blood-agar plates (figs. 23 and 24)

Morphology: Long slender rods, usually partly decolorized by gram stain

Culture 441. Isolated September 19, 1924, at postmortem, from a case of gangrene of the retroperitoneum. This culture corresponds

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Fig. 20. Clostridium botulinum type b (No. 178 Hall)
Double method, forty-eight hour culture. × 6

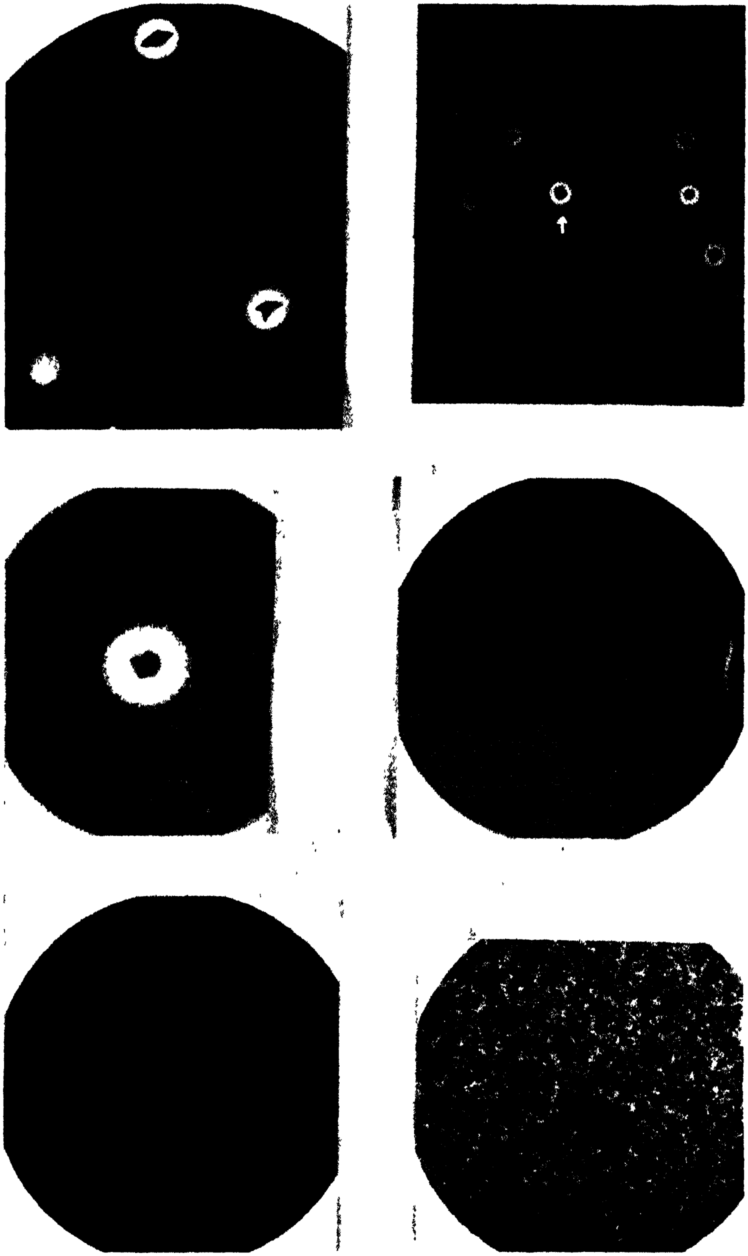
Fig. 21. Clostridium sporogenes (No. 90 Hall)
Double method, forty-eight culture. × 2

Fig. 22. Clostridium sporogenes (No. 90 Hall)
Double method, forty-eight hour culture. × 6

Fig. 23. Clostridium tertium (No. 518 Hall)
Single method, twenty-four hour culture. × 2

Fig. 24. Clostridium tertium (No. 518 Hall)
Single method, twenty-four hour culture. × 6

Fig. 25. Clostridium putrificum (No. 38 Hall)
Double method, six-day culture. × 20



morphologically and culturally with *Clostridium tertium*, and gives the same effect on blood-agar plates as the preceding culture

Culture 542–2. Isolated November 23, 1924, at postmortem from the lung in a case of terminal bronchopneumonia. All cultural characters are like those given above. In addition to these strains of *Clostridium tertium* other anaërobes producing green zones on blood agar have been isolated. As yet they have not been identified with any species described at present

Clostridium putrificum

Culture 38. (I. C. Hall) Six days' incubation

Growth was slow and was obtained only by the double method

Surface colony: None

Deep colony: Very tiny lens-shaped or oval compact masses

Hemolysis: None (fig. 25)

This culture grows very slowly in any medium. It is a representative of the nonhemolytic group. Other members of this group have been encountered in routine anaërobic culturing, but thus far they have not been identified with any described species. It is hoped that in time enough of them may be collected to make possible a comparative study.

SUMMARY AND CONCLUSIONS

The anaërobic blood-agar plate, when used as described, offers a very good general means of isolating and cultivating spore-forming anaërobes. It is also an aid in judging the purity of cultures.

Three groups of the anaërobes can be made, classified according to their effect on red blood cells: (1) the hemolytic group, comprising all of the well known pathogenic forms investigated in this work; (2) the group producing methemoglobin, or green zone colonies, and (3) that without effect on hemoglobin.

The blood-agar plate offers a rapid method of judging the significance of spore-forming anaërobes which may be encountered in clinical bacteriology, since the common pathogenic forms are hemolytic.

As an additional cultural method for identification of anaërobes, the blood-agar plate should be as valuable, if not more valuable, than any one of the test media commonly employed, such as brain broth, coagulated egg broth, and litmus milk.

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THE EFFECT OF LACTIC ACID BACTERIA ON THE ACETONE-BUTYL ALCOHOL FERMENTATION[1]

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In the manufacture of solvents by the fermentation process, Thaysen (1921) and Speakman and Phillips (1924) have shown that lactic acid bacteria often cause serious losses. The antagonism between *Granulobacter pectinovorum*[2] and the lactic acid bacteria is usually so marked that within twelve to eighteen hours after inoculation the growth of the acetone-butyl alcohol organism is suppressed.

In previous papers (Fred et al., 1925, and Stiles et al., 1925) the occurrence and the fermentation characteristics of some of these harmful lactic acid bacteria from corn mash have been discussed.

According to their effect on the acetone-butyl alcohol fermentation, these lactic acid bacteria may be divided into three groups: first, long rod forms usually granulated and very injurious to the butyl alcohol organism, second, long and short rod forms which may or may not be granulated, but never with large distinct granules as seen in group one, and which are also harmful to the butyl alcohol organism, but not so injurious as the preceding group; third, rod forms often very small, almost cocci, which are harmless or only slightly injurious to the growth of the butyl alcohol organism. These lactic acid organisms may be divided in other ways according to their fermentation products. For example, nos. 2, 3, 4, 7, 15, 19, 20 and 21, in table 1, produce

[1] Published with the Approval of the Director of the Agricultural Experiment Station.

[2] For convenience the term Granulobacter or Granulobacter pectinovorum is used throughout this paper in place of Bacillus granulobacter pectinovorum.

large amounts of lactic acid and very little CO_2 in the breaking down of sugars, while nos. 5, 6, 8, 9, 10, 11, 12, 13, 14, 16, 17 and 18 produce small amounts of acid, mannitol from fructose, ethyl alcohol from aldo-hexoses, and considerable amounts of CO_2 from these sugars.

The present communication deals with the harmful effect of the lactic acid bacteria on the butyl alcohol organism, and also with some of the factors that favor the growth of these lactic acid bacteria in mashes. Two phases of the subject have received special study; the persistence of the various lactic acid bacteria in mashes, and the nature of the agent injurious to the butyl alcohol-producing organism.

EXPERIMENTAL

The cultures of the lactic acid bacteria used in this study were isolated from mash of contaminated fermenters of the acetone-butyl alcohol fermentation. From more than two hundred cultures obtained from mash, fifteen of the more distinctive strains were selected for special study. In addition to the organisms from corn mash, five cultures of lactic acid bacteria isolated from other sources were used. Through the kindness of the Curator of the Lister Institute, London, transfers of the original culture of *Bacterium volutans*, Fleming and Thaysen, were supplied. Although this culture had been kept in the laboratory for several years, it had not lost its ability to injure *Granulobacter pectinovorum*. This culture from the Lister Institute will be discussed under the name, *Bacterium volutans*.

Some of the cultures have been under study for almost three years, and during this time have never shown a permanent loss in toxicity.[3] Occasionally the lactic acid bacteria develop in mash without producing any harmful effect on the growth of *Granulobacter pectinovorum*. Subsequent transfers from this same culture, however, have always shown that this apparent loss of toxicity is only temporary. The so-called non-toxic

[3] The term toxicity as used in this paper relates to the injurious effect of lactic acid bacteria on the butyl-alcohol organism and not to any specific substance elaborated by the former.

strains of the granulated lactic acid organism have not been encountered, although variations in degree of toxicity are not uncommon. If transfers are not made at frequent intervals, many of these high acid-forming bacteria are soon lost. Attempts to carry these cultures on such media as peptone agar or beef peptone agar, with or without sugar, proved unsatisfactory. The best results were obtained from cultures grown on potassium phosphate glucose yeast-water agar.

Effect of lactic acid bacteria on the growth of Granulobacter pectinovorum in corn mash

The inhibiting effect of the lactic acid bacteria on the growth of *Granulobacter pectinovorum* is quickly and easily determined from corn mash cultures inoculated with these two organisms. In a corn meal mash, the growth of *Granulobacter pectinovorum* may easily be detected by its amylolytic activity, as indicated by the decrease in viscosity, and opaqueness of the medium. In addition to these changes, the growth of the butyl alcohol organism results in a vigorous gas production, and also a compact head consisting of protein, fibrous material and slime. This head is forced to the top of the liquid in the early stages of the fermentation, and remains as a thick compact mass. The lactic acid bacteria on the other hand produce very little gas and no head. In the presence of certain strains of lactic acid bacteria, the characteristic gas and head of the butyl alcohol fermentation are lacking, and most of the mash remains as an unfermented residue in the bottom of the flask.

To measure the antagonism between the lactic acid and butyl alcohol bacteria, small Erlenmeyer flasks containing 100 cc. of 8 per cent corn mash were inoculated with 1 cc. of a twenty-four-hour old culture of *Granulobacter pectinovorum* and an equal quantity of a lactic acid culture. The gas and head formation were noted and at the end of three days the titratable acid and pH value determined. The results are shown in table 1. From the figures of this table it is clear that many of the lactic acid bacteria inhibit markedly the growth of the butyl alcohol organism, while others exert very little effect. Those forms commonly found in

water infusions of cereals, such as *L. leichmanni*, *L. mannitopoeum*, *Bact. volutans*, and *L. gracile* are especially harmful to the growth of *Granulobacter pectinovorum*. An exception to this is noted with

TABLE 1

Showing the effect of lactic acid bacteria on the growth of Granulobacter pectinovorum and on the acid produced in corn mash

Eight per cent corn mash

NUMBER	CULTURE NAME OR NUMBER	GROWTH OF GRANULOBACTER IN THE PRESENCE OF VARIOUS STRAINS OF LACTIC ACID BACTERIA		REACTION AFTER THREE DAYS	
		Growth	Head	0.1 N acid in 10 cc. of culture	H ion
				cc.	*pH*
1	Control*	Good	Good	3.5	5.4
2	*L. leichmanni*	Poor	None	12.2	3.6
3	*L. leichmanni*	Poor	None	11.9	3.6
4	*L. leichmanni*	Poor	None	11.3	3.7
5	*L. mannitopoeum*	Poor	None	10.9	3.7
6	*L. mannitopoeum*	Poor	None	10.0	3.8
7	*Bact. volutans*†	Poor	None	10.0	3.7
8	*L. mannitopoeum*	Poor	None	9.7	3.7
9	*L. mannitopoeum*	Poor	None	9.0	3.8
10	*L. gracile*	Poor	None	9.0	3.8
11	*L. gracile*	Poor	None	8.8	3.8
12	*L. intermedium*	Fair	Fair	7.9	3.8
13	*L. intermedium*	Fair	Fair	5.6	3.9
14	Culture 19	Fair	Good	4.7	5.0
15	Culture 60	Good	Good	4.7	5.2
16	Culture 7	Good	Good	4.5	5.2
17	Culture 4	Good	Good	3.8	5.2
18	*L. pentoaceticus*	Good	Good	3.8	5.2
19	*Streptococcus lactis*	Good	Good	3.7	5.5
20	*L. bulgaricus*	Good	Good	3.5	5.4
21	*L. acidophilus*	Good	Good	3.4	5.4

* *Granulobacter pectinovorum* alone.

† *Bacterium volutans* probably identical with *L. leichmanni*.

L. pentoaceticus and cultures nos. 4, 7 and 19. Culture no. 60, a streptococcus, has no effect on the growth of *Granulobacter pectinovorum*. The results suggest that the inhibition of the solvent-forming organism is related to the amount of acid produced.

Relation between the size of inoculum and the amount of acid formed

Five hundred cc. portions of corn mash in Erlenmeyer flasks were inoculated with an active *Granulobacter pectinovorum* culture and also with varying amounts of the lactic acid organism. At regular intervals samples were drawn from these flasks and the reaction measured.

TABLE 2

Effect of Lactobacillus leichmanni on acid production in corn mash inoculated with Granulobacter pectinovorum

NUMBER	AGE	GRANULOBACTER ALONE		GRANULOBACTER PLUS 1 CC. OF L. LEICHMANNI		GRANULOBACTER PLUS 5 CC OF L. LEICHMANNI		GRANULOBACTER PLUS 10 CC. OF L. LEICHMANNI	
		Acid*	pH	Acid*	pH	Acid*	pH	Acid*	pH
		cc.		*cc.*		*cc.*		*cc.*	
1	After Beginning	0.2	6.6	0.2	6.6	0.3	6.5	0.3	6.4
2	3 hours	0.4	6.0	0.5	5.6	0.6	5.4	0.8	5.0
3	6 hours	0.8	5.3	1.3	4.8	1.3	4.6	1.5	4.4
4	9 hours	2.0	5.0	2.4	4.8	2 4	4.4	2.3	4.4
5	12 hours	3.5	4.8	4.1	4.4	3 9	4.0	3.1	4.0
6	15 hours	3.9	4.6	4.8	4.4	4.1	4.0	4.2	4.0
7	18 hours	4.2	4.4	5.1	4.2	4.5	3.8	4.4	3 8
8	21 hours	2.9	4.2	7.6	3.7	4.7	3.6	4.9	3 6
9	24 hours	2.5	4.6	8.4	3.6	5.0	3.6	5.0	3.6
10	27 hours	2.2	4.8	9.0	3.6	5.2	3.6	5.1	3.6
11	30 hours	2.0	4.8	9.3	3.4	5.8	3.6	5.4	3.6
12	3 days	3.2		11.4		10.0		9.8	
13	7 days	4.1		13 0		10.8		10.4	
14	14 days					11.7		11.4	
15	30 days	4.4		13.5		11.7		11.4	

* 0.1 N acid in 10 cc. of culture.

The formation of titratable acid and change in hydrogen ion concentration during the fermentation are given in table 2. Within three hours after inoculation, the flasks which received lactic acid bacteria showed a great increase in the hydrogen ion concentration. This gain in active acidity was especially noticeable in the cultures which received the largest amounts of lactic acid culture. By the end of six hours this rapid increase in the dissociated acid reached a pH of 4.8 or below while the

control containing *Granulobacter pectinovorum* alone did not exceed pH 5.3. The low buffer content of corn mash and consequent high dissociation of acids in all probability soon renders the mash unsuitable for the growth of the solvent-forming organism. The highest titratable acid occurred where small rather than large inocula of the lactic acid bacteria were used. High production of titratable acid is the result of a fine balance between the growth of the acetone-butyl alcohol organism and the lactic acid bacteria in the early stages of fermentation. If one or the other

TABLE 3

Relation between age of lactic acid cultures and their inhibiting effect on Granulobacter pectinovorum

In 5 per cent corn mash

NUMBER	ORIGINAL CULTURE	KIND OF GRANULOBACTER FERMENTATION				
		Age in days of the original culture added to corn mash				
		2	7	14	21	42
1	*Granulobacter* plus *L. leichmanni*	None	Poor	Poor	Fair	Good
2	*Granulobacter* plus *L. leichmanni*	None	Poor	Fair	Fair	Good
3	*Granulobacter* plus *L. intermedium*	Poor	Fair	Fair	Fair	Good
4	*Granulobacter* plus *L. intermedium*	Poor	Fair	Fair	Fair	Good
5	*Granulobacter* plus *L. mannitopoeum*	Poor	None	None	None	None
6	*Granulobacter* plus *L. mannitopoeum*	Poor	None	None	None	None

completely dominates the fermentation, then it is impossible to secure a high acid production. The maximum acid production was obtained with a 1 per cent inoculum of *Granulobacter pectinovorum* and a 0.2 per cent of *L. leichmanni*. In other words, to secure a high total acidity of the mash, the combined action of the two organisms is required. These data will be discussed in connection with another experiment.

Although the total acidity was measured after three, seven, fourteen and thirty days no marked gain in acid was found after the third day. The two cultures which received the larger

amounts of *L. leichmanni* did show a decided increase in acid after the thirtieth hour.

Attempts to favor the growth of one of these groups of organisms, and thus overcome the other, by incubating at various temperatures was tested repeatedly. Without exception, the lactic acid organisms soon dominated the fermentation.

The persistence of lactic acid bacteria in corn mash

Laboratory studies have shown that in the usual culture media many of the lactic acid bacteria, especially the high acid formers, exist for only a short time. Whether or not this same condition obtains in such natural substrates as corn mash is not known. It seems probable that these organisms will persist for a much longer time in cereal mashes. To secure an answer to this question a large number of tests with various strains of the lactic acid bacteria, and with various media, have been carried out. Only a summary of the more important points will be presented.

Flasks containing 300 cc. of a 5 per cent corn mash were inoculated with different strains of the lactic acid bacteria, *Lactobacillus leichmanni*, *Lactobacillus intermedium*, and *Lactobacillus mannitopoeum*. Twelve hours later, 30 cc. of the acetone-butyl alcohol organism were added to each flask. *L. leichmanni* is typical of the high acid, non-mannitol-forming, rod-shaped, granulated lactic acid bacteria possessing decided toxic properties; the other two organisms are small rods, representative of the low acid, mannitol-forming bacteria, and are not so toxic to *Granulobacter pectinovorum*. At first all of the cultures showed gas production, especially the flasks inoculated with *L. intermedium* and *L. mannitopoeum*. At the end of twenty-four hours the flasks which received *L. leichmanni* ceased to show gas, and a large deposit of unfermented starch remained. The other cultures gave a better fermentation, and only a small amount of unfermented starch remained.

To test for the presence and the toxicity of these lactic acid bacteria, samples were drawn from the original stock flasks and seeded into tubes of sterilized corn mash, as follows: 1 cc. portions of these mixed cultures were added to duplicate tubes of

20 cc. each of corn mash, and twelve hours later 1 cc. of an active culture of the acetone-butyl alcohol organism was added.

When forty-eight hours old, enrichment cultures were made (that is, transfers to fresh tubes of mash), and an active culture of *Granulobacter pectinovorum* was added. The results of these enrichment cultures, which were carried out simply as confirmatory tests, to eliminate the possibility that the change in reaction due to the inoculum might be the cause of the injury to the butyl alcohol organism, are not recorded in the tables.

The results obtained from the associated growth of the two kinds of organisms in fresh mash are given in table 3. The horizontal columns on the right of this table ("kind of granulobacter fermentation") show the toxic effect of the lactic acid bacteria on the butyl alcohol fermentation. The terms used in this table, for example "none," mean that transfers from the original culture into fresh mash and then seeded with fresh cultures of Granulobacter failed to give any growth. For each test after two, seven, fourteen, twenty-one and forty-two days old, fresh vigorous cultures of the acetone-butyl alcohol organism were added.

The results show definitely that in the mashes of this experiment the toxicity of *L. leichmanni* decreases with an increase in age until, after twenty-one to forty-two days, no injury is noted. The *L. intermedium* culture failed to show any decided change in toxicity after the second day. Apparently this member of the lactic acid family is not very harmful to the growth of *Granulobacter pectinovorum.*

In relation to time, *L. mannitopoeum* behaves in a manner entirely different from the high acid former *L. leichmanni;* the toxicity increased rather than decreased in the older cultures. The formation of neutral substances, alcohol instead of lactic acid, probably accounts for the longevity of this culture in mash.

Additional evidence on the longevity of the lactic acid bacteria is given in table 4. Here the procedure differed from that given in the foregoing experiment. The amount of active culture of *Granulobacter pectinovorum* was reduced from 10 per cent to 2.0 per cent, and also the amount of the lactic acid inoculum was

greatly reduced. Instead of allowing a period of incubation, both cultures were added at the same time. This change in method of inoculation resulted in quite different fermentations. The butyl alcohol organism showed a most active fermentation for more than forty-eight hours, and but little starch was left undigested.

TABLE 4

Relation between age of and different treatments of lactic acid cultures and their inhibiting effect on Granulobacter pectinovorum

In 5 per cent corn mash

NUMBER	KIND AND AMOUNT OF INOCULUM		ORIGINAL FERMENTATION (HEAD)	REACTION AFTER 1 MONTH (0.1 N ACID IN 10 CC. OF CULTURE)		KIND OF GRANULOBACTER FERMENTATION — Age in days of the original lactic acid cultures added to corn mash		
	Organism	Per cent		*cc.*	*pH*	60	160	365
1	*Granulobacter* alone	2.00	Good	4.5	4.1	Good	Good	Good
2	*L. leichmanni* alone	0.02	None	1.3	4.4	None	None	None
3	*Granulobacter* plus *L. leichmanni*	2 00 0.02	Good	4.4	3.9	None	None	None
4	*L. intermedium* alone	0.02	None	0.8	4.4	Poor	Fair	Fair
5	*Granulobacter* plus *L. intermedium*	2.00 0.02	Good	3.8	4 2	Poor	Fair	Fair
6	*L. mannitopoeum* alone	0.02	None	0.6	4.5	None	Poor	None
7	*Granulobacter* plus *L. mannitopoeum*	2.00 0.02	Good	8.0	3 7	None	Poor	Poor

As shown by Peterson, Fred and Domogalla (1924), the *Granulobacter pectinovorum* fermentation increases the buffer content of the medium. This gain in buffer, which is caused by the increase in soluble nitrogenous compounds, and the production of acids of low dissociation, coupled with the decrease in fermentable sugar, should bring about conditions favorable to the persistence of the lactic acid bacteria. The results shown in Tables

3 and 4 support this statement. The original *Granulobacter pectinovorum* fermentations recorded in table 3 were stopped long before the carbohydrate was consumed, while in table 4 these fermentations continued until almost all of the carbohydrate was destroyed. This difference in the original *Granulobacter pectinovorum* fermentation no doubt accounts for the long persistence of the lactic acid bacteria in the mashes of table 4.

TABLE 5

The production of lactic acid in mixed cultures of Granulobacter pectinovorum and L. leichmanni

Calculated for 1 liter of mash

NUMBER	KIND AND AMOUNT OF INOCULUM		0.1 N ACID IN 10 CC.	SOLVENTS	ZINC LACTATE	
	Organism	Per cent			Weight	Water of crystalization*
			cc.	*grams*	*grams*	*per cent*
1	*Granulobacter* alone	1.0	3.3	12.7	0.0	
2	*Granulobacter* plus *L. leichmanni*	1.0 0.05	4.0	11.2	1.53	16.7
3	*Granulobacter* plus *L. leichmanni*	1.0 0.25	4.6	10.5	2.25	15.1
4	*Granulobacter* plus *L. leichmanni*	1.0 0.5	7.1	0.2	4.84	15.5
5	Pancreatin and *L. leichmanni*	1.0	6.3	0.0	2.97	13.0

* Theory for inactive lactic acid, 18.2 per cent; for the active form, 12.9 per cent.

The presence of the lactic acid bacteria in the old mash was traced by microscopic examinations. When the cultures were two hundred and three hundred sixty-five days old, isolation plates were poured and the organisms secured in this way compared with the original stock lactic acid cultures. No differences in fermentation reactions or toxicity between the old and the new subcultures were noted.

An experiment similar to the foregoing was carried out with the

stock cultures kept at 37°C. instead of room temperature, 20 to 22°C. Since it was assumed that the organisms would probably die much quicker at the higher temperature, samples were drawn every five days and tested for the presence of harmful organisms. The lactic acid bacteria were alive regardless of the medium in which the cultures had been kept, whether mash fermented with Granulobacter or unfermented mash. These tests were discontinued after thirty days.

Solvent and lactic acid production in mixed cultures of Granulobacter pectinovorum and L. leichmanni

In a mixed culture the rôle played by each microörganism can be followed chemically by determining the product most characteristic of each: for Granulobacter, the solvents, acetone, and butyl alcohol; for *L. leichmanni*, lactic acid. A fermentation in which Granulobacter predominates will be high in solvents and low in lactic acid, and the reverse will be true where *L. leichmanni* is the dominating factor. It must be borne in mind, however, that the lactic acid organism can not grow to any extent upon the unmodified corn mash, and a suitable medium for its development depends upon the amylolytic and proteolytic action of Granulobacter. By varying the amount of inoculum in a series of flasks, fermentations grading from an essentially Granulobacter to that of a chiefly *L. leichmanni* type may be secured. The data from such a series are given in table 5. In order to make certain that lactic acid was being measured, the zinc salt was prepared and its water of crystallization was determined. The data show that solvents and lactic acid are inversely proportional to one another. As the quantity of *L. leichmanni* used in the inoculum was increased, solvent production decreased and the amount of zinc lactate increased.

An interesting result of the associated action of these bacteria is the effect on the relative quantity of d and l lactic acid produced. As shown in a previous publication *L. leichmanni* produces in pure culture almost entirely levo-lactic acid. In the case of no. 5, which was a pure culture of *L. leichmanni*, the water of crystallization is 13.0 per cent and is very close to that for the

levo enantiomorph. In nos. 2, 3, and 4, which were mixed cultures of the two bacteria, the water of crystallization shows the production of a larger quantity of dextro-acid by *L. leichmanni* than in no. 5. The effect of association on the forms of lactic acid and other products will be dealt with more fully in a later publication.

The nature of the inhibiting agent

The product of the lactic acid-forming organism, responsible for the injurious effect on the solvent-forming bacteria, has not been definitely identified, although the subject has been studied and much discussed. In view of our knowledge of the harmful influence exerted by acid-producing organisms on their associates, it would seem that we should look first to acidity as the cause of this antibiotic relation.

Speakman (1920) showed that during a normal fermentation of corn mash by *Granulobacter pectinovorum* a typical curve of acidity may be traced, which consisted of three phases. There is observed a rapid rise in the production of acid to a maximum. This is followed by a decided fall to a minimum and finally a slow rise from the minimum. Reilley and others (1920) have shown that the acids accumulated during the first phase of the curve are chiefly acetic and butyric with evidence of a third unknown kind. Schmidt, Peterson and Fred (1924) have since proved leucic acid to be present. Thaysen (1921) pointed out that a "serious infection" greatly modifies the normal acid curve as to time and extent of rise and fall. Such modifications are accompanied by irregularities in the entire fermentation, showing usually a rise of acidity above the maximum. Reilley and his associates state in their publication, that such erratic curves of acidity are associated with large amounts of lactic acid. The bacteria most commonly found to be responsible for the changes here noted belong to the lactic-acid-producing group. In all the earlier discussions at hand concerning this interference with the work of *Granulobacter pectinovorum* wherever reference is made to the influence of contamination, it seems to have been taken for granted that the results were due to the acidity produced by the

foreign organisms. More recently, however, doubt has been expressed as to the validity of this belief. On the basis of work recently done, Speakman and Phillips (1924) contend that the effect can not be due to the acid produced by the lactic acid bacterium, and they adopt the tentative hypothesis that the inhibitory agent is a product of the nitrogen metabolism of the contaminating organism. Scarcity of experimental evidence on this point and lack of agreement as to the part played by the acid produced by the lactic acid bacteria, appeared to warrant a more intensive laboratory study of this problem.

Some factors that influence acid production in corn mash inoculated with the lactic acid bacteria alone

As reported by Speakman and Phillips (1924) all attempts to secure high acid production in corn mash alone inoculated with these contaminating forms of lactic acid bacteria failed. When glucose was used and Sorensen's phosphate added to the medium as a buffer substance the result was entirely different. Under the above condition the lactic acid bacteria form large amounts of titratable acid as shown in table 6. In the absence of *Granulobacter pectinovorum*, but in the presence of a buffer and of glucose, *L. leichmanni* produces a fairly high concentration of acid. The results show that the presence of a fermentable carbohydrate, like glucose, is not sufficient to bring about acid production in mash. It is essential that a buffer be present to take care of the free acidity. A similar result can be accomplished by pre-digesting the corn mash with pancreatin. A sufficient quantity of available carbohydrates and protein is thus produced and the buffer capacity of the medium is increased. In such a medium *L. leichmanni* produced a considerable quantity of lactic acid. From 500 cc.of culture, 1.47 gm. of zinc lactate with 13.04 per cent water of crystallization was obtained. Corn mash media are unsuitable for the growth of *L. leichmanni* in several respects: lack of fermentable sugar, low buffer capacity and possibly insufficient soluble protein and phosphates. If these deficiencies are corrected, no difficulty is experienced in bringing about growth of the organism and acid production.

TABLE 6

The formation of acid in corn mash inoculated with Lactobacillus leichmanni alone

4 per cent corn mash

NUMBER	TREATMENT	TITRATABLE ACID AFTER						
		At beginning	1 day	2 days	3 days	5 days	10 days	15 days
		cc †	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
1	Mash alone	0.25	0.50	1.0	1.2	1.2	1.2	1.4
2	Mash plus 0.2 per cent phosphate*	0 70					3.0	3.2
3	Mash plus 2.0 per cent glucose	0.30	1.4	1.7	2.1	2.2	2.2	2.2
4	Mash plus 2.0 per cent glucose 0.2 per cent phosphate	0.75	3 6	5.2	6.2	6.6	6.8	7.4
5	Mash plus 2.0 per cent glucose 0.5 per cent phosphate	1.65	3.6	5.0	6.0	6.6	7.1	7.7
6	Mash plus 2.0 per cent glucose 1.0 per cent phosphate	2.35	3.2	4.8	6.0	7.4	7.5	8.2
7	Mash predigested with Takadiastase	0.40			1.0	1.1	1.7	
8	Mash predigested with pancreatin	0.50			5.9	6.3		

* Sorenson's phosphate $Na_2HPO_4 + 2\ H_2O$ was used.

† 0.1 N acid in 10 cc. of medium.

TABLE 7

The influence of the products produced by L. leichmanni in corn mash on the growth of the butyl-alcohol organism

5 per cent corn mash plus 1 per cent of glucose

NUMBER	AGE OF LACTIC ACID CULTURE	TREATMENT	GROWTH OF GRANULOBACTER IN MASH PREVIOUSLY INOCULATED WITH THE LACTIC ACID BACTERIA			REACTION
			Growth	Gas	Head	
	hours					*pH*
1	24	Sterilized	Good	Profuse	None	5.8
2	24	Neutralized and sterilized	Good	Profuse	Good	
3	48	Sterilized	Fair	Slight	None	4.7
4	48	Neutralized and sterilized	Good	Profuse	Good	
5	72	Sterilized	None	None	None	4.5
6	72	Neutralized and sterilized	Good	Profuse	Good	

The effect of heat and neutralization of acids on the fermentation of corn mash by Granulobacter pectinovorum

The following experiments were arranged with reference only to the rôle played by acids in the antagonistic effect on Granulobacter. A direct comparison was made of the true acidity produced by the lactic-acid bacteria and of the corresponding acidity produced by additions of pure acids to the culture medium. After sterilization, the lactic acid bacteria were inoculated into the flasks and allowed to grow for various lengths of time. At the end of the period specified, the cultures were heated sufficiently to kill the lactic acid organism. Five flasks of each group were then inoculated with a vigorous culture of *Granulobacter pectinovorum*, and development recorded as shown in the accompanying tables. A sixth flask in each group was reserved for the determination of titratable acidity, and the pH value produced by the bacteria.

The outline of this experiment and the results obtained are shown in table 7. While the lactic acid bacteria alone in corn mash plus glucose do not produce as high a titratable acid as is found in mixed cultures containing *Granulobacter pectinovorum*, the hydrogen-ion concentration is not greatly different. It is well-known that a pH value of 4.8 to 4.6 is unfavorable to *Granulobacter pectinovorum;* and that pure cultures of the lactic acid bacteria will, in glucose mash, produce a value even lower than these figures.

The effect of the acid produced by these lactic acid bacteria in preventing the growth of the *Granulobacter pectinovorum* is clearly shown from the figures of this table. If old mash cultures of the lactic acid bacteria are inoculated with Granulobacter, there is no sign of growth. However, when these same cultures are neutralized, the butyl-alcohol organism develops. This fact has been noted where *Granulobacter pectinovorum* had been dormant for as long as eight days. The mash culture was merely neutralized, and no new inoculation of Granulobacter made.

Additional data concerning the nature of the toxic agent were obtained from cultures which had been seeded with *L. leichmanni*

and *Granulobacter pectinovorum.* Within three days after inoculation these cultures reached a maximum acidity of 11.5 cc. of 0.1 N acid in 10 cc. These cultures were kept for two weeks, but did not show any gain in total acidity beyond that found on the third day. As found by transfers to fresh media, the lactic acid bacteria under the conditions of this test were entirely destroyed before the end of one week. The complete destruction of the lactic acid bacteria in these cultures offered an opportunity to measure the *Granulobacter pectinovorum* fermentation in highly toxic cultures in which the acidity may be neutralized but the cultures not subjected to the heat of sterilization. To carry out this test large flasks of the mash were neutralized, inoculated with a fresh culture of Granulobacter, and a representative sample removed for solvent analysis. The fermentation in this neutralized but unheated mash was not as vigorous as commonly noted in fresh mash. However, by the second day there was a strong gas production and well formed head. The analysis for solvents of this 6 per cent mash is shown below:

	Total solvents in 1000 cc. of culture
	gm.
At the end of second fermentation neutralized and reinoculated....	8.88
At beginning of second fermentation..........................	2.11
Gain..	6.77

According to the conditions of this test, neutralization alone without heat removes the toxic agent from corn mash cultures. The evidence points strongly to acidity as the prime cause in the injury to the butyl-alcohol fermentation.

That the lactic acid radical is not only non-toxic to Granulobacter, but is actually destroyed by the organism, was proved by adding calcium lactate (0.12 to 0.24 per cent) to the corn mash before inoculation, and analyzing the culture at the end of the fermentation. Such cultures, if anything, gave a better head and gas production than the controls. A careful chemical analysis failed to show any lactic acid in the flasks containing the smaller amount of calcium lactate and only a trace in those to which the larger quantities had been added.

Effect of varying amounts of organic and inorganic acids.

In tables 8 and 9 are given the results of this study. The relation between hydrogen-ion concentration and growth of *Granulobacter pectinovorum* is the important point brought out by these

TABLE 8

The influence of varying amounts of organic acids on the growth of Granulobacter pectinovorum

In 5 per cent corn mash

NUMBER	KIND OF ACID	1 N ACID	REACTION	KIND OF GRANULOBACTER FERMENTATION		
				Growth	Gas	Head
		per cent	pH			
1	Lactic	0.2	5.7	Good	Profuse	Good
2	Lactic	0.4	5.6	Good	Profuse	Good
3	Lactic	0.6	5.6	Good	Profuse	Good
4	Lactic	0.8	5.1	Good	Profuse	Good
5	Lactic	1.0	4.8	Good	Medium	Fair
6	Lactic	1.2	4.7	Fair	Slight	None
7	Lactic	1.4	4.6	None	None	None
8	Lactic	1.6	4.3	None	None	None
9	Acetic	0.2	5.9	Good	Profuse	Good
10	Acetic	0.6	5.8	Good	Profuse	Good
11	Acetic	1.0	5.4	Good	Profuse	Good
12	Acetic	1.6	5.0	Good	Profuse	Good
13	Acetic	2.2	4.8	Fair	Medium	Good
14	Acetic	2.6	4.7	Fair	Slight	None
15	Acetic	3.0	4.6	Poor	None	None
16	Butyric	0.2	6.0	Good	Profuse	Good
17	Butyric	0.6	5.7	Good	Profuse	Good
18	Butyric	1.0	5 1	Good	Profuse	Good
19	Butyric	1.6	4.9	Good	Profuse	Good
20	Butyric	2.2	4.8	Fair	Medium	Fair
21	Butyric	2.6	4.7	Poor	Slight	None
22	Butyric	3.0	4.6	Poor	Slight	None
23	Butyric	3.2	4.5	None	None	None

tests. It is not the percentage of the acid but the pH value which determines its inhibiting effect on the butyl-alcohol fermentation. The mineral acids are even more effective in preventing growth of *Granulobacter pectinovorum* than the organic acids. This injurious effect varies slightly among the acids

tested. As seen in table 9 *Granulobacter pectinovorum* will grow in slightly lower pH with hydrochloric than with either sulfuric or phosphoric acids, but the difference is not great. Since *Granulobacter pectinovorum* has the power to destroy these organic acids, and thus reduce the hydrogen-ion concentration, it is to be expected that the organism will start at a slightly higher

TABLE 9

The influence of varying amounts of inorganic acids on the growth of Granulobacter pectinovorum

In 5 per cent corn mash

NUMBER	KIND OF ACID	1 N ACID	REACTION	KIND OF GRANULOBACTER FERMENTATION		
				Growth	Gas	Head
		per cent	*pH*			
1	Sulphuric	0.1	6.0	Good	Profuse	Good
2	Sulphuric	0.2	5.8	Good	Profuse	Good
3	Sulphuric	0.4	5.6	Fair	Medium	None
4	Sulphuric	0.6	5.2	None	None	None
5	Sulphuric	0.8	5.0	None	None	None
6	Hydrochloric	0.1	5.8	Good	Profuse	Good
7	Hydrochloric	0.2	5.6	Good	Profuse	Good
8	Hydrochloric	0.4	5.4	Good	Medium	None
9	Hydrochloric	0.6	5.2	Fair	Medium	None
10	Hydrochloric	0.8	5.0	Poor	None	None
11	Hydrochloric	1.0	4.8	None	None	None
12	Phosphoric	0.1	6.0	Good	Profuse	Good
13	Phosphoric	0.2	5.9	Good	Profuse	Good
14	Phosphoric	0.4	5.8	Good	Profuse	Good
15	Phosphoric	0.6	5.7	Good	Medium	Fair
16	Phosphoric	0.8	5.6	Fair	Slight	None
17	Phosphoric	1.0	5.4	Fair	Slight	None

acidity when this is due to organic acids, than when it is caused by unfermentable compounds.

The results here reported have reference only to the pH value necessary to prevent initiation of growth of the butyl-alcohol organism. Nothing has been ascertained as to the pH value which would check growth when once under way. It would no doubt be lower than that required to prevent the beginning of development, as Granulobacter is known to produce a very con-

siderable buffer effect when active in a medium. In mixed cultures, the development appears always to be in favor of the lactic acid-forming organism. In fact some hydrolysis of starch in the corn mash is necessary before the lactic acid organism has a suitable supply of carbohydrate upon which to act. During this initial growth, the Granulobacter would probably form the buffers mentioned.

The non-toxic effect of filtrates of cultures of L. leichmanni in the Granulobacter fermentation

The question has been asked, Is the antagonistic effect of the lactic acid bacteria due to non-acid substances which pass through a filter? To find an answer to the question, a great number of filtration experiments were carried out. Cultures of the lactic acid bacteria of various ages were passed through Berkefeld and Pasteur Chamberland filters of varying porosity. The bacteria were removed in this way and the neutralized filtrate added to corn mash plus *Granulobacter pectinovorum.* All attempts, however, to suppress the growth of the acetone-butyl-alcohol-forming organism with these filtrates failed.

Similar results were secured from experiments with collodion sacks. Here the different cultures were grown in the same medium separated only by the thin membrane. If it is assumed that the bacteria produce inhibiting substances which will diffuse through a membrane, then the harmful effect of the lactic acid cultures should be readily detected by the use of the special culture tube for collodion sacks (Mulvania, 1924). Here again, no injury other than that common to an acid reaction was ever noted.

SUMMARY

In the mash inoculated with *Granulobacter pectinovorum,* the common types of cereal lactic acid bacteria grow rapidly producing large amounts of lactic acid.

The formation of the lactic acid in the mashes is dependent upon the associated action of the two groups—the lactic acid and the butyl-alcohol organisms. The presence of the lactic acid

bacteria alone is not sufficient. The growth of *Granulobacter pectinovorum* favors the development of the lactic acid bacteria in several ways; by hydrolysis of the starch to fermentable carbohydrates, by proteolysis of the nitrogen compounds to amino acids—production of buffer substances. Although this association is beneficial to the lactic acid bacteria, it is extremely harmful to the solvent-forming organism.

These harmful lactic acid bacteria may be divided into various groups. The high acid-forming organisms described under the name *Lactobacillus leichmanni* are the most injurious to the butyl-alcohol fermentation. Next in order of their inhibiting effect, belong the organisms described as mannitol-formers, *Lactobacillus mannitopoeum*, *Lactobacillus gracile*, and *Lactobacillus intermedium*.

The persistence of the lactic acid bacteria in mash for long periods of time depends on the kind of organism and also on the degree of the associated growth with Granulobacter. The high acid-producing bacteria such as *L. leichmanni* persist for only a short time if they have had an abundance of fermentable sugar and available nitrogen. Quite the contrary is true of the mannitol-forming group of lactic acid bacteria; these usually produce much smaller amounts of acid and survive for a much longer period. In the mash alone, without *Granulobacter pectinovorum*, the lactic acid bacteria persist for at least one year, perhaps for a longer time. The presence of a protective colloid, starch, and the presence of only a small amount of fermentable sugar no doubt favor the longevity of the lactic acid bacteria. Strange to say, somewhat similar results in relation to longevity of lactic acid bacteria are obtained when the *Granulobacter pectinovorum* produces a very vigorous fermentation. Here also the conditions favorable to longevity of the lactic acid bacteria are present; namely, large amounts of buffer substances and relatively small amounts of acid. The life of the lactic acid bacteria is comparatively short, provided the growth of *Granulobacter pectinovorum* is sufficient only to hydrolyze much of the starch, but not to carry it through to the formation of neutral substances, and thus large amounts of acid are formed and only small amounts of buffer substances.

There is no evidence to indicate that lactic acid is more harmful to the growth of *Granulobacter pectinovorum* than the other organic or inorganic acids. When additions of pure acetic, lactic, butyric, sulphuric, hydrochloric and nitric acids are added to mash in amounts sufficient to give pH values of about 4.7 to 4.8, there is a well-defined inhibiting effect on the growth of the acetone-butyl alcohol organism. If this acidity is neutralized, the injurious property is removed. Similar results are obtained with mashes in which the acidity is produced by the lactic acid bacteria, provided the mashes are kept until all of the living lactic acid bacteria have died off. The evidence, taken as a whole, points to acidity as the chief factor injurious to the growth of *Granulobacter pectinovorum*.

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CONDITIONS AND REACTIONS DEFINING DYE BACTERIOSTASIS

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The continued study of bacterial cells from the point of view which assumes that they behave as conjugated proteins (Stearn and Stearn, 1924a and b) has led to an intensive study of the action of both basic and acid dyes in their bacteriostatic and staining reactions.

The use of buffers in bacteriological media is becoming more and more common, since it is generally conceded that bacteria have very decided pH limits of growth unless slow acclimatization beyond these limits has taken place. Little of the existing data on bacteriostatic agents has taken into account that the effective dilution of these agents varies greatly with the pH of the media, and since the pH fluctuates as growth proceeds it is absolutely necessary to have sufficient buffer present for its control.

Browning, Gulbransen and Kennaway (1920) found that the sterilizing effect of diamino-acridine methyl chloride is multiplied one hundred times by a change of pH from 4 to 11. There are, however, few known organisms which have natural pH limits of growth as wide as these authors suggest. In studying the pH effect on bacteriostatic action it is essential to remain well within the limits of growth, preferably within the optimum range of H-ion concentration.

I. J. Kligler (1918) in his study of bacteriostasis by dyes attempted to control the pH of the media by the addition of 0.5 per cent K_2HPO_4. This gives an initial pH of 7.1 and protects the media against formation of acids during growth, but has no buffering power against bases. Even then this is by no means

the mostly highly effective H-ion concentration for bacteriostatic action by basic dyes. According to Dernby (1921) there are organisms such as the staphylococcus, whose optimum H-ion concentration lies between the pH limits of 7.2 and 7.6. For such an organism our data here presented will show that basic dyes would be far more inhibitive at a pH of 7.6 than at 7.1—the former being incidentally the pH of human blood.

Traube (1912) found that Na_2CO_3 accentuates the toxicity of certain stains, notably crystal violet. Prowazek (1910) noted likewise that the addition of Na_2CO_3 increased the activity of methylene blue. Dernby and Davide (1923) state that eucupin and the quinine alkaloids are more effective against the staphylococcus and diphtheria bacilli in solutions which are more alkaline than the blood.

In the cases of acidic substances, Graham-Smith (1919) found quinine more effective in acid than in neutral or alkaline solutions. Davis and White (1918) found that acid chlor-mercury fluorescein was more active in acid solution.

Data showing to what extent it is important to determine the H-ion concentration at which a specific bacteriostat is most inhibitive for a specific organism are given below, which suggest answers to other relative and cogent questions. The pH range through which the effect of any dye was studied was not arbitrarily chosen but was the range through which the organism in question has been shown to grow. Except where otherwise stated these limits were obtained from Dernby (1921). In all cases the limits of the range were tested by growing the particular strains studied in 0.2 per cent lactose broth adjusted to the upper and lower limiting values of the particular range through which they were subsequently to be studied. The media were buffered by phosphate mixtures. A minimum total salt concentration of about one-fifteenth molar was required to hold the pH constant. This latter was checked both before and after incubation by the indicator method. Doubling the concentration of buffer material did not affect the results. In the cases where gentian violet was used a 0.2 per cent lactose broth was generally employed. It was found, however, that identical results were obtained when a 1 per cent glucose broth was used in its stead.

A. BASIC DYES

As Simon and Wood (1914) have suggested, we may assume that in the structure of the bacterial organism we find receptors for either acidic or basic substances. This idea might be represented by a type formula $R\frac{NH_2}{COOH}$, the acid group acting as receptor for a basic substance and vice versa; or it might be more simply represented by the formula HPrOH.

The present authors (1923) found that at a high pH range, from about 7.6 to 8.5, bacteria as they developed in gentian violet media would settle to the bottom as a deep purple precipitate, decolorizing the solution, whereas at a lower pH range, from 4.3 to 5, the bacteria which settled were only slightly stained and the solution remained a deep purple.

The following series of equilibria may be thought of as establishing themselves in a system of bacteria and dye. We will represent any basic dye by the formula DOH.

(1) $DOH \leftharpoondown\!\!\rightharpoonup D^+ + OH^-$

(2) $HPrOH \rightleftharpoons H^+ + PrOH^-$

(3) $D^+ + PrOH^- \rightleftharpoons DPrOH$

(4) $DPrOH + H_2O \leftrightharpoons HPrOH + D^+ + OH^-$

Maximum bacteriostatic action occurs with maximum formation of the un-ionized dye-protein compound, DPrOH. A study of the above equilibria would lead, among others, to three predictions. Data bearing on each of these are presented below.

1. The long arrows in the above set of equations show the direction in which the equilibrium would shift for an increase in alkalinity. This direction is obvious for equations 1 and 2, and it is clear that the effect of hydroxyl ion concentration on number 3 is only indirect. Equation 4, the hydrolysis of the dye-protein compound, takes account of the fact that the dye will be much stronger as a base than the bacterial cell protein is as an acid, and thus for comparison of effect the dye is represented as ionized compared to the protein. Increase in alkalinity would aid, directly or indirectly, the formation of DPrOH, and thus

augment the bacteriostatic effect of a basic dye, in the equilibria represented by equations 2 and 4. It would work against the formation of DPrOH in equation 1. Since, however, the dye will be much more strongly ionized than the protein the effect of an increase in alkalinity on number 1 will be much less than on number 2,[1] and we should expect an increase in alkalinity to increase the effective dilution of the basic dye.

TABLE 1

BACILLUS DYSENTERIAE (SHIGA)
LIMITING pH RANGE 6.2 TO 7.6

Gentian violet dilution	pH			
	5.58	6.23	7.16	7.73
45,000	–	–	–	–
50,000	+	–	–	–
60,000	+	–	–	–
100,000	+	+	–	–
200,000	+	+	+	+

BACILLUS TYPHOSUS
LIMITING pH RANGE 5.4 TO 9.1

Gentian violet dilution	pH			
	6.23	6.81	7.16	7.73
150,000	–	–	–	–
200,000	+	+	–	–
300,000	+	+	+	–
500,000	+	+	+	+
No dye	+	+	+	+

BACILLUS COLI
LIMITING pH RANGE 4.4 TO 7.8

Gentian violet dilution	pH			
	5.28	6.23	7.16	7.73
10,000	–	–	–	–
20,000	+	–	–	–
30,000	+	+	–	–
70,000	+	+	–	–
100,000	+	+	+	–
200,000	+	+	+	+

BACILLUS AEROGENES
OPTIMUM pH 6.5*

Gentian violet dilution	pH			
	5.2	6.23	7.1	7.7
5,000	–	–	–	–
10,000	+	–	–	–
30,000	+	+	–	–
50,000	+	+	+	–
100,000	+	+	+	+

* Smith (1922).

Table 1 gives the results of experiments with gentian violet on some Gram-negative organisms. Twenty-four hour agar

[1] An example will make this clear. If we take two weakly ionized substances, one weaker than the other, say with ionization constants 10^{-5} and 10^{-9}, and calculate the effect of a change of pH from 4.0 to 8.0, we find, assuming for simplicity that they are both bases, that the ion concentration of the stronger is decreased by 10 ten times while that of the latter is decreased by a million times. I.e., the same pH change affects the weaker one 10,000 times as much as the stronger in the above case, which is fairly typical.

cultures were washed off by the addition of sterile nutrient broth. To insure the same amount of inoculation two drops of bacterial suspension were transferred by means of a sterile pipette to each tube of 0.2 per cent lactose broth containing definite amounts of dye and buffered with a phosphate mixture. The results are those obtained after seventy-two hours incubation.

Table 2, giving results for strongly Gram-positive organisms, while in accord with the well known fact that this group of organisms is in general more sensitive to basic dyes than the Gram-negative shows clearly that the behavior toward these dyes under varying conditions is perfectly analogous to that of the Gram-negative organisms. The culture of staphylococcus used was isolated from an infected knee and was found to grow

TABLE 2

STAPHYLOCOCCUS AUREUS LIMITING pH RANGE 5.6 TO 8.1					STREPTOCOCCUS HEMOLYTICUS LIMITING pH RANGE 5.5 TO 8.0					
Gentian violet dilution	pH				Gentian violet dilution	pH				
	6.4	7.0	7.2	7.6		6.4	7.1	7.3	7.7	8.0
2,000,000	–	–	–	–	4,000,000	+	–	–	–	–
3,000,000	+	–	–	–	5,000,000	+	+	+	–	–
4,000,000	+	+	+	–	6,000,000	+	+	+	+	–
6,000,000	+	+	+	+	8,000,000	+	+	+	+	+
					10,000,000	+	+	+	+	+

in a concentration of gentian violet 1:2,000,000 at a pH of 6.4 even though that was beyond its optimum limits of growth. The growth limits are given as 5.6 to 8.1 but the optimum lies between 7.2 and 7.6.

Here also the data represent the results of seventy-two hours incubation.

Table 3 gives results obtained with two other basic dyes brilliant green, which is more strongly basic than gentian violet, and para-rosaniline, which is less strongly basic than gentian violet. The organism studied was *Bacillus coli*. Data for both forty-eight and seventy-two hours incubation are included. The peculiar behavior of the brilliant green should be especially noted. Its behavior was normal up to a pH of about 7, beyond

which its color faded out. Up to the point of this change it exerts an extremely powerful bacteriostatic action, but beyond this point this power is largely lost. The point is rather sharp since at 6.8 to 6.9 perfectly normal action is encountered while at 7.16 peculiar results are obtained.

Another striking phenomenon, which, unfortunately, it is impossible to show in our tables, is the *comparative quantity* of growth. In general it may be said that in such cases as repre-

TABLE 3

BRILLIANT GREEN					PARA-ROSANILINE				
Dye dilution	pH				Dye dilution	pH			
	4.95	5.28	6.46	7.16		5.28	6.23	7.16	7.73
50,000	−	−	−	−	5,000		(See note)		
	−	−	−	−					
75,000	−	−	−	−	7,500	+	+	−	−
	+	−	−	+		+	+	−	−
150,000	+	+	−	−	10,000	+	+	−	−
	+	+	−	+		+	+	−	−
300,000	+	+	+	−	15,000	+	+	+	+
	+	+	+	+		+	+	+	+
600,000	+	+	+	+					
	+	+	+	+					

Note: At this concentration there was growth in all tubes as evidenced by slight precipitation. This was very slight at the lower values of pH gradually increasing to the higher.

sented in table 1 for those concentrations of dye where there is growth in all tubes at all H-ion concentrations studied, the amount of growth falls off as the alkalinity increases, as one might expect from the remainder of the table. These "gradations" in quantity of growth with the pH are almost as striking as the "gradations" in the limiting dilution of dye with the pH, and furnish an equally striking substantiation of the prediction as to the pH effect.

II. Reversibility. The equilibria represented in equations 1 to

4 above should be reversible. As already pointed out (Stearn and Stearn, 1924b) the bacteria are probably not killed by these dyes except perhaps at fairly high concentrations, but are merely fixed and rendered temporarily powerless to multiply. The following is the evidence that this prediction is true.

Tubes containing dye in which no growth appeared after seventy-two hours incubation were treated with a solution containing the same concentration of dye but so acidified that the final pH would be lower than that at which the original incubation took place, and would be one at which decided growth had been shown to take place. Invariably, after twenty-four hours incubation, growth took place, shown not only by clouding of the media but also by streaking on agar and microscopically

TABLE 4

pH OF ORIGINAL BROTH (NO GROWTH OCCURRED)	APPROXIMATE pH OF ADJUSTED BROTH (GROWTH AFTER TWENTY-FOUR HOURS)	DYE DILUTION	ORGANISM
7.3	6.0	50,000*	*B. coli*
8.0	6.0	100,000*	*B. coli*
7.0	6 0	10,000*	*B. aerogenes*
7.7	6.0	7,500†	*B. coli*

* Gentian violet.
† Para-rosaniline.

examining the growth. This guaranteed that the cloudiness of the broth was due to the original organisms themselves which had again manifested their reproducing power, and not to the presence of a contaminating form. Experiments were made with both gentian violet and para-rosaniline. Table 4 includes a few of the many experiments performed along this line. In the case of the adjusted broth the control of the pH is not quite as close as in the other cases.

III. Basic strength of dye. A third prediction one might make is that the greater the basic strength of the dye the more effective a bacteriostat it will be at any one pH. This is brought out in equation 1, for the stronger the dye the greater the concentration of dye ion D^+. While no accurate data on the ionization constants of the dyes used are available, the effect on the basic or

acidic strength of the substitution of various groups in various positions in the molecule is more or less definitely known, and thus comparative basic strengths can be determined. (For a somewhat more full discussion of this question applied to the triphenylmethane dyes, see Stearn and Stearn, 1924b.) Table 5 compares the limiting dilutions of the basic dyes used on *Bacillus coli* at the same pH. The order is that of decreasing basic strength. It should be pointed out that from consideration of their formulas there is probably little difference between the basic strength of gentian violet and methyl violet. They should be nearly the same. The former has in it one more methyl group and might be a shade the stronger.

TABLE 5

DYE	LIMITING DILUTION		
	pH 5.2	pH 6.2	pH 7.7
Brilliant green	200,000	250,000	Power destroyed
Gentian violet	15,000	25,000	150,000
Methyl violet	10,000	15,000	70,000
Para-rosaniline	5,000	5,000	10,000

B. ACID DYES

A set of equilibria, analogous to those given for basic dyes, could be given for acid dyes. From such a set the three analogous predictions might be made. Data bearing on these points are presented below. These data are not as extensive as in the case of basic dyes but the general behavior is indicated almost as conclusively. Perhaps the most important feature regarding these data, one which has been mentioned but which should be emphasized, is that in this work the authors have limited their range of study to conditions of solutions in which the organisms are known to thrive in the absence of dye, so that the data are less equivocal than much of the material at present available in this field.

1. Increase in alkalinity with acid dyes should have the

opposite effect to that with basic dyes. Table 6 gives results for eosin, acid violet 5B, and acid fuchsin. Using a buffered 0.2 per cent lactose broth, a loopful of *Bacillus coli* suspension was inoculated and incubated for seventy-two hours. In the more alkaline solutions the dyes are seen to be less effective.

TABLE 6

DILUTION	pH						
	5.28	6.23	7.16	7.73	4.95	6.23	7.15
Acid fuchsin							
25	−			+			
50	−	−	+	+			
75	−	−	+	+			
100	−	+	+	+			
Acid violet							
25					−		+
50					−	−	+
100					−	+	+
200					+	+	+
Eosin							
25	−			+			
50	−	+	+	+			
75	−	+	+	+			
100	+	+	+	+			

TABLE 7

pH OF ORIGINAL BROTH (NO GROWTH OCCURRED)	pH OF ADJUSTED BROTH (GROWTH IN TWENTY-FOUR HOURS)	DYE DILUTION	ORGANISM
6.0	7.0	100*	*B. coli*
6.0	7.0	200*	*B. coli*

* Acid fuchsin.

II. Reversibility. As in the case of basic dyes, it was possible to take a tube in which no growth had occurred after seventy-two hours incubation, and, by adding alkaline dye solution, keeping the concentration of dye constant, to obtain vigorous growth in twenty-four hours. Table 7 is analogous to table 6.

III. Acidic strength of dye. Table 6 is arranged in the probable order of decreasing acidic strength of the dyes. A glance at these data will show the increase in bacteriostatic power with the acidic strength, keeping the pH constant.

C. EFFECT OF THE PREVIOUS ENVIRONMENT OF THE ORGANISM ON ITS SENSITIVITY TO DYES

The importance of this problem, from the point of view both of classification or identification and of resistance to bacteriostats and bacteriocides, is apparent. Below are presented the results of a few preliminary experiments in this direction. They will not be discussed at this time.

I. A twenty-four-hour growth of *Staphylococcus aureus* was stained for one minute with a sterilized solution of gentian violet by thoroughly mixing while still suspended on the agar slant. The stained organisms were then inoculated into nutrient broth, one tube at a pH of 6.2 and another at a pH of 7.7. After twenty-four hours incubation vigorous growth of the organism had developed in the broth at a pH of 6.2, but there was no growth whatsoever in the other tube.

II. For two weeks a culture of *Bacillus coli* grew in nutrient broth whose pH was kept at 6.2. Another culture was grown at a pH of 7.7. They were twice transferred to new media and kept at 37°. At the end of this period a loopful of the culture media was inoculated into methyl violet—0.2 per cent lactose broth. The results are included in table 8. Readings were made after twenty-four, forty-eight, and seventy-two hours. While the final readings were the same in the case of both cultures, the twenty-four-hour and even the forty-eight-hour readings were not. The culture which had been kept in the more alkaline medium at a pH of 7.7 is rendered temporarily more sensitive to the action of the methyl violet, and it is only after some time that it recovers its normal ability to multiply in the presence of the dye.

The results under "a" were from the culture kept for two weeks in a broth at a pH of 6.2, those under "b" were from the culture at 7.7 described above. The results under "c" came

from inoculations from a suspension prepared from an agar slant kept at a pH of about 7.0 for forty-eight hours.

TABLE 8

DILUTION OF METHYL VIOLET	pH											
	5.2			6.2			7.1			7.7		
	a	b	c	a	b	c	a	b	c	a	b	c
10,000	+	−	−	−	−	−	−	−	−	−	−	−
	+	+	−	−	−	−	−	−	−	−	−	−
	+	+	+	−	−	−	−	−	−	−	−	−
20,000	+	+	−	+	−	−	−	−	−	−	−	−
	+	+	+	+	−	−	−	−	−	−	−	−
	+	+	+	+	+	+	−	−	−	−	−	−
30,000	+	+	+	+	+	+	−	−	−	−	−	−
	+	+	+	+	+	+	−	−	−	−	−	−
	+	+	+	+	+	+	−	−	−	−	−	−
70,000	+	+	+	+	+	+	+	+	−	+	−	−
	+	+	+	+	+	+	+	+	−	+	+	−
	+	+	+	+	+	+	+	+	+	+	+	−
100,000	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+
200,000	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+

D. SUMMARY AND DISCUSSION

1. In defining the bacteriostatic strength of a dye it is necessary to state the pH at which it is to act.

2. A method of proving the reversibility of a dye bacteriostatic reaction has been shown. This indicates an added reason why it is essential to give repeated injections of gentian violet in its use intravenously.

3. For a series of organisms not too widely different in character, the same concentration of the same dye might easily be

given as the limiting inhibitive dilution merely by altering the reaction of the medium. This is brought out in table 9 using data obtained with gentian violet.

4. In several cases, with Gram positive organisms, unexpected results were obtained. For instance in certain series in which *Streptococcus hemolyticus*, isolated from the spinal fluid in a case of meningitis, was being studied, it was found that at a pH of 5.9 and in a concentration of gentian violet 1:2,000,000 growth occurred. Upon examination the culture proved to contain long chains of streptococci about half of which were Gram positive and the remainder distinctly and strongly Gram negative. Even after subculturing on agar chains of the cocci would exhibit this characteristic; i.e., a deeply stained blue coccus would be united to a deeply stained red one, etc. The growth

TABLE 9

ORGANISM	pH	
	Limiting dilution, 50,000	Limiting dilution, 100,000
Bacillus aerogenes	7.73	8 plus
Bacillus coli	7.16	7.73
Bacillus typhosus	6.81	7.16
Bacillus dysenteriae	Less than 5.0	Less than 5.0

in this particular tube seemed quite instructive and peculiar since no such thing had taken place at even higher dilutions of gentian violet and higher values of pH. Similar results were obtained with *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus subtilis*. Growth of any of these distinctly Gram positive organisms in scattered tubes of large series we found meant the presence of strains of so-called mutants which might develop other unlooked for characteristics. The study of these mutating strains is now in progress, and with it a study of the effective cause which stimulated this pronounced and peculiar action.

In conclusion it is a pleasure to acknowledge the suggestions of Dr. B. F. Sturdivant, Director of the Laboratories of the Pasadena Hospital, whose continued interest has been a source of inspiration.

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CELLOBIOSE AS AN AID IN THE DIFFERENTIATION OF MEMBERS OF THE COLON-AEROGENES GROUP OF BACTERIA[1]

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INTRODUCTION

The very general acceptance of the presence of colon bacilli in water supplies as an index of more or less recent fecal pollution has made the correct delimitation and ready identification of the species a matter of great importance.

More than thirty years ago, Theobald Smith (1890), pointed out that colon bacilli could be subdivided into two sub-groups according to their action upon sucrose. From that period to the present, many workers have interested themselves in the differentiation and classification of the species and subspecies of this group.

Of the twenty-two different species of colon-like organisms listed in the 1923 edition of Bergey's Manual, twelve produce acid and gas from sucrose while ten do not. On a statistical basis it is said that approximately 40 per cent of the strains of colon bacilli split sucrose. The recognition and significance of the non-sucrose fermenting strains presents no difficulty; those strains which utilize sucrose, however, present the constant danger of confusion with the aerogenes types which are also frequently present in water supplies.

The colon bacillus is a commensal inhabiting the intestinal

[1] An abstract of this paper was originally presented at the Ithaca Meeting of the American Chemical Society, September 1924.

tract of man and other vertebrates and except under very unusual conditions is incapable of multiplication as a free living saprophyte,—therefore its presence elsewhere may be assumed to be indicative of fecal pollution of human or animal origin.

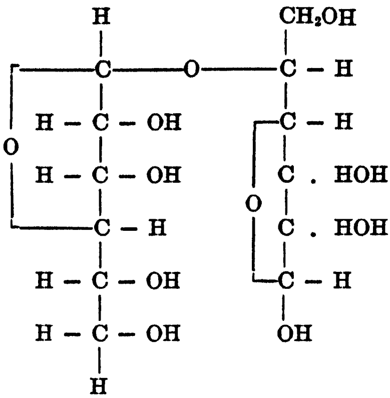
The aerogenes bacillus on the other hand, while also frequently present in the intestine is a free living saprophyte widely distributed in nature. Its presence, therefore, in water is not indicative of fecal pollution. Hence the differentiation of sucrose fermenting strains of colon bacilli from strains of the aerogenes type is both of academic interest and of practical importance.

At present the grouping of a strain may be based on several physiological tests, no one of which is of itself entirely conclusive. The formation of acetyl-methyl-carbinol as determined by the Voges-Proskauer (1898) test, and the ability to grow in Koser's (1918) uric-acid medium, are criteria commonly used. Another criterion, usually termed the "methyl red test" (Clark and Lubs, 1915) depends on the differences in H-ion concentration which are caused by the action of *E. coli* and *A. aerogenes* on *d-glucose* broth. Very recently B. H. Butcher (1924) of Iowa has proposed the use of chinic acid and Koser of Illinois has suggested the use of citric acid media for the differentiation. Of these tests, the V.-P. test for acetyl-methyl-carbinol enjoys at present the official sanction of the committee of the S. A. B. and the generic classification into the *Genera Escherichia* and *Aerobacter* is based on the results of this test.

Chen and Rettger (1920) and others (see bibliographies of Chen and Rettger 1920 and Rogers), have shown that all of these tests are misleading in certain cases and this fact is so generally recognized that critical differentiation is always based on correlated evidence.

The present paper deals with a test which from studies so far completed appears to differentiate sharply between *E. coli* and *A. aerogenes*. It depends upon the use of a sugar, *cellobiose*, which was first obtained from cellulose by Skraup and Koenig, (1901) and which has proved of marked interest to investigators of the constitution of cellulose. The researches of Haworth and Hirst (1921) and of Bergmann and Schotte (1921) have shown

clearly that cellobiose is a glucosido—5-glucose having the following structure:



The fact that cellobiose is hydrolyzed by emulsin indicates that the sugar is a β-glucoside.

While cellobiose is ordinarily obtained in the form of its octaacetate in the acetolysis of cellulose, it has also been reported by Pringsheim (1923) among the hydrolytic products obtained from cellulose by the action of various microörganisms including the denitrifying, methane and hydrogen producing bacteria, as well as the thermophylic cellulose bacteria and cellulose destroying fungi.

Evidently all of these organisms elaborate a *cellulase* which functions over a wider range of temperature than does the accompanying *cellobiase* (which hydrolyzes cellobiose into two molecules of *d*-glucose). In the case of thermophylic bacteria, Pringsheim carried out his hydrolytic fermentation of cellulose at 67°, and was able to arrest the degradation of cellulose at the cellobiose stage without causing it to be hydrolyzed further to glucose.

Despite the great theoretical interest attached to cellobiose we have found no evidence that it has been utilized in practical bacteriological work.

We have found as a new and interesting property of cellobiose that the sugar is rapidly utilized by *A. aerogenes* with acid and gas

formation and that no such changes are brought about by any strains of *E. coli* examined by us. On the basis of these differences in behavior our test for distinguishing between *E. coli* and *A. aerogenes* has been devised. It involves no new technic, is very simple, and (for the strains examined) absolutely conclusive. The method for the preparation of cellobiose, and the procedure and tabulated results are given in the experimental part of this paper.

EXPERIMENTAL PART

The cellobiose[2] used in these experiments was prepared from cellobiose octaacetate. The latter was obtained from cellulose (cotton cellulose or filter paper) by an acetolysis that was practically identical with that described by Madsen (1917) and which has been given in detail by Wise and Russell (1922).

The conversion of the octaacetate was carried out by gradually stirring 20 grams of the cellobiose octaacetate into 150 cc. of a cold 10 per cent solution of potassium hydroxide in 95 per cent alcohol. The white crystalline monopotassium derivative was filtered, washed with absolute alcohol and dissolved in ice water. This alkaline solution was then immediately titrated to the neutral point with dilute acetic acid, filtered and concentrated nearly to dryness in *vacuo*. The residue was then crystallized from ethyl alcohol or methanol and was usually found to be sufficiently pure for our purposes. It could be further purified by solution in water and precipitating by the addition of acetone.

Needless to say, all precautions must be taken to avoid hydrolysis of the disaccharide to glucose but in general these conditions are easily met. Resinification of the sugar (especially in alkaline solution) must also be avoided. The cellobiose used in our experiments was a colorless or faintly tinted microcrystalline powder, melting at 225°C. (uncorrected).

[2] The cellobiose used in our experimental work was prepared by Mr. Floyd Peterson, graduate assistant in Forest Chemistry at the New York State College of Forestry. Our thanks are due to Mr Peterson for his enthusiastic coöperation throughout the course of this work. His results on the preparation of cellobiose will be published elsewhere.

The cellobiose medium used was prepared by the addition of 0.5 per cent of the sugar to nutrient broth containing 0.3 per cent Difco beef extract and 1 per cent Difco pepton. Sterilization was carried out by autoclaving for 20 minutes at approximately 121°C. after distribution in fermentation tubes of either the Smith or the Dunham type. Results were observed after twelve, twenty-four, forty-eight and one hundred and twenty hours incubation at 37°. Obviously the sugar may be added to any sort of liquid or liquefiable media just as other sugars are, and in the same proportions. There is such an abundant gas production, however, in a broth containing 0.5 per cent that there would seem to be no advantage in using more and probably a much smaller proportion would be equally satisfactory.

Tables 1 and 2 indicate the results obtained with known strains of *E. coli* and *A. aerogenes*. It will be noted that in every case the strains of *A. aerogenes* have split the cellobiose with gas and acid production, while neither acid nor gas was produced by *E. coli*.

It will be noted that with the 52 organisms in the series, the Voges-Proskauer test correlates with the cellobiose test in every case with the exception of *E. neopolitana* 602 (Series I). The ability of this strain of *E. neopolitana* to utilize cellobiose may be interpreted as circumstantial evidence that the strain is an aberrant species of the genus Aerobacter.

It will be noted also that the organism designated as *A. aerogenes* S. gives a positive methyl-red test with a positive Voges-Proskauer and a positive reaction with cellobiose.

The results obtained in these studies seem to indicate that the use of cellobiose affords a means of differentiating the genera Escherichia and Aerobacter that is fully equal to those now in use in dependability and superior to them in simplicity and in the ease of interpretation.[3]

[3] The cultures used in Series I were supplied by Dr. L. F. Rettger of Yale University and Dr. M. Levine of the University of Iowa, to whom our thanks are due.

TABLE 1

Series 1. Reactions of a series of stock strains of the colon-aerogenes group on cellobiose

ORGANISM	SOURCES	VOGES-PROSKAUER TEST	METHYL-RED TEST	CELLOBIOSE
E. coli 434	Human feces	Negative	Positive	Negative
E. coli 436	Human feces	Negative	Positive	Negative
E. coli 442	Human feces	Negative	Positive	Negative
E. coli 446	Human feces	Negative	Positive	Negative
E. coli 460	Human feces	Negative	Positive	Negative
E. coli 465	Human feces	Negative	Positive	Negative
E. coli S	Sheep feces	Negative	Positive	Negative
E. coli C	Cow feces	Negative	Positive	Negative
E. coli H	Horse feces	Negative	Positive	Negative
E. coli CH	Chicken feces	Negative	Positive	Negative
E. coli M	Monkey feces	Negative	Positive	Negative
A. cloacae		Positive	Negative	Acid and gas (25 per cent)
A. aerog.	Human feces	Positive	Negative	Acid and gas (50 per cent)
A. aerog.	Rat feces	Positive	Negative	Acid and gas (50 per cent)
A. aerog. 1	Soil	Positive	Negative	Acid and gas (35 per cent)
A. aerog. 3	Soil	Positive	Negative	Acid and gas (40 per cent)
A. aerog. S	Soil	Positive	Positive	Acid and gas (50 per cent)
E. coli La		Negative	Positive	Negative
E. coli 24		Negative	Positive	Negative
E. paragrunthali 36		Negative	Positive	Negative
E. communior 41		Negative	Positive	Negative
E. coscoroba 70		Negative	Positive	Negative
E. acidi-lac. 43		Negative	Positive	Negative
E. acidi-lac. 165		Negative	Positive	Negative
E. neapolitani 594		Negative	Positive	Negative
E. neapolitani 602		Negative	Positive	Acid and gas (50 per cent)
A. cloacae 233	Soil	Positive	Negative	Acid and gas (95 per cent)
A. cloacae 235	Human feces	Positive	Negative	Acid and gas (95 per cent)
A. aerog. 252	Soil	Positive	Negative	Acid and gas (75 per cent)
A. aerog. 80	Soil	Positive	Negative	Acid and gas (50 per cent)

TABLE 2

Series 2. Reactions of a series of colon-aerogenes strains on cellobiose

ORGANISM	SOURCE	VOGES-PROSKAUER TEST	METHYL-RED TEST	SUCROSE	CELLOBIOSE
E. coli 1	Human feces	Negative	Positive	Negative	Negative
E. coli 2	Human feces	Negative	Positive	Negative	Negative
E. coli 3	Human feces	Negative	Positive	Negative	Negative
E. coli 4	Human feces	Negative	Positive	Negative	Negative
E. coli 5	Human feces	Negative	Positive	Negative	Negative
E. coli 6	Human feces	Negative	Positive	Acid and gas	Negative
E. coli 7	Human feces	Negative	Positive	Acid and gas	Negative
E. coli 8	Human feces	Negative	Positive	Negative	Negative
E. coli 9	Human feces	Negative	Positive	Negative	Negative
E. coli 10	Human feces	Negative	Positive	Negative	Negative
E. coli 11	Horse feces	Negative	Positive	Negative	Negative
E. coli 12	Horse feces	Negative	Positive	Negative	Negative
E. coli 13	Horse feces	Negative	Positive	Negative	Negative
E. coli 14	Horse feces	Negative	Positive	Negative	Negative
E. coli 15	Horse feces	Negative	Positive	Acid and gas	Negative
E. coli 16	Water	Negative	Positive	Acid and gas	Negative
E. coli 17	Water	Negative	Positive	Acid and gas	Negative
A. aerog. 18	Human feces	Positive	Negative	Acid and gas	Acid and gas (50 per cent)
A. aerog. 19	Water	Positive	Negative	Acid and gas	Acid and gas (75 per cent)
A. aerog. 20	Water	Positive	Negative	Acid and gas	Acid and gas (30 per cent)
A. aerog. 21	Water	Positive	Negative	Acid and gas	Acid and gas (50 per cent)
A. aerog. 22	Horse feces	Positive	Negative	Acid and gas	Acid and gas (50 per cent)

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THE ELECTROPHORETIC MIGRATION OF VARIOUS TYPES OF VEGETABLE CELLS[1]

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PREVIOUS KNOWLEDGE IN REGARD TO THE ELECTROPHORETIC MIGRATION OF VEGETABLE CELLS

During the latter part of the nineteenth century a number of more or less elementary observations were made in regard to the behavior of protozoa under the influence of electrical currents—work which is summarized by Dale (1901). Following the studies of Hardy (1899; 1900) on the influence of electrical charge upon the coagulation of colloidal particles, more active progress was made in the study of this problem and the work was extended to cover other unicellular organisms. Fischer (1900) described the effect of weak currents upon the infusoria and predicted that bacteria would behave in a similar fashion, although, because of their minute size, experimentation in this case would be more difficult. Bechhold (1904) was apparently the first, actually to observe and record the fact that bacterial cells do carry a negative charge. Lillie (1903) determined the direction of migration of various isolated cells in the path of a current and stated that cells with abundant nuclear material migrated to the anode, as did silica dust, while cells with voluminous cytoplasm, such as certain red blood cells, leucocytes and muscle cells travelled with the positive stream. The latter result has not been confirmed by later workers. Greeley (1904) reported exhaustive studies in regard to the migration of protozoa which brought out the influence of acidity and alkalinity upon the direction of movement.

[1] Thesis presented by the junior author in candidacy for the degree of Doctor of Philosophy at Yale University.

Neisser and Friedemann (1904) observed the negative charge on bacterial cells. Teague and Buxton (1906) discovered that an increase in hydrogen ion concentration causes a decrease in charge and that with continuing increase in acidity the charge is ultimately reversed so as to become positive. (See also Buxton and Shaffer, 1906.) Cernovodeanu and Henri (1906) studied a larger series of bacteria than any of their predecessors and reported that all of the species except *B. dysenteriae* (Flexner), as well as a mold, carried a negative charge. The claim that the dysentery bacillus is electro-positive has since been contradicted by Szent-Györgyi (1921). The forms of apparatus used by many of these earlier investigators (generally involving macroscopic observation of the aggregation of bacteria at one or the other end of a U-tube) did not lend themselves to exact quantitative results. A glass cell adapted to more accurate microscopic studies was introduced by Lortet (1896) used by Russ (1909) and Thornton (1910) and perfected by Powis (1914) and by Northrop (1922). Hardy and Harvey (1911) first employed non-polarizable electrodes in work of this kind.

Many important studies on the electrophoretic migration of bacteria have been conducted during the past few years with the use of the technique thus devised. A fairly extensive bibliography has been presented elsewhere by Winslow, Falk and Caulfield (1923); and it seems unnecessary to review the literature again in detail. Three communications of some significance have, however, appeared since the paper to which reference has just been made (Eggerth, 1923, 1924; Winslow and Shaughnessy, 1924; Winslow and Fleeson, 1925).

The net result of the work which has been done to date may be briefly summed up as follows:

A. Unicellular organisms (whether living or dead), as well as certain inorganic particles, bear a negative charge. Abbott (1908) and Thornton (1910), Cernovodeanu and Henri (1906)—in the case of *B. dysenteriae* only, and Szent-Györgyi (1921)—in the case of a fungus from the stomach of a mouse—report positive charges but the evidence on the other side is, in general, so strong as to make it likely that these observers were in error.

It is quite probable that they may have been misled by the phenomenon of electrical endosmose in the vicinity of the glass.

B. The negative charge on the cell is increased by very slight additions of certain neutral salts (Winslow and Fleeson, 1925).

C. The charge on the cells is markedly decreased with higher concentrations of salts or with the addition of acid or alkali (Northrop and DeKruif, 1922; Winslow, Falk and Caulfield, 1923; Winslow and Fleeson, 1925).

D. The charge is reversed and becomes positive with very high concentration of acids or alkalies or with some bivalent and trivalent salts (Northrop and DeKruif, 1922; Winslow and Shaughnessy, 1924; Winslow and Fleeson, 1925).

E. The effects noted under (C) are solely, or at least predominantly, due to the cation (Winslow and Fleeson, 1925).

F. In general, the effect of the cation increases with its valency but hydrogen is more active than other monovalent ions (Northrop and DeKruif, 1922; Winslow and Fleeson, 1925).

G. The presence of a monovalent ion tends to neutralize the effect of a bivalent ion and the presence of a positive ion other than hydrogen tends to some extent to neutralize the influence of the hydrogen ion itself (Winslow, Falk and Caulfield, 1923).

OBJECTIVES OF THE PRESENT INVESTIGATION AND TYPES OF ORGANISMS STUDIED

The object of the present study was to discover the extent to which the reactions noted above could be discovered to be specific for various types of vegetable cells. Certain previous workers had observed one or two organisms with care and others had recorded merely qualitative results, or results at certain limited reaction ranges for a larger series of forms. It was our purpose to select a dozen or more vegetable microörganisms, representing widely separated biological types, and to study their electrophoretic migration under a wide range of pH values in order to determine how far in the words of Loeb (1922) the phenomena of cataphoretic charge are due to "forces inherent in

the water itself" and how far they are affected by the specific physical and chemical properties of the suspended cell.

In the conduct of these studies we used three fundamental menstrua, distilled water, Ringer's solution and a solution of 0.145 M NaCl (isotonic with Ringer's solution), observations being made in each of the three menstrua at reactions between pH 1.5 and pH 12.5.

Complete tests were made in this fashion with twelve different organisms and with silica dust particles; and in addition, the results obtained with our primary strain of streptococcus were checked by observations at a few typical pH values of four additional strains of streptococci, obtained from diverse sources and possessing diverse biological attributes.

The thirteen primary series of experiments were conducted with the following types of organisms (or inorganic suspended particles):

1. Particles of silica dust. Finely divided particles of silica dust from material used by Dr. Leonard Greenburg in electrophoretic studies. They ranged from 6 to 10 microns in size.

2. Chlorella. A one-celled green alga obtained from Dr. F. B. Wann of the New York State College of Agriculture, Cornell University, labelled no. 11. This organism grew readily on one per cent glucose agar in sunlight at room temperature. Its cells were about 6 microns in diameter.

3. Saccharomyces apiculatus. A typical small yeast obtained from the Department of Botany of the Michigan Agricultural College. It grew readily on glucose agar at room temperature. Its cells were about the same size as those of Chlorella and as the particles of the silica dust. This is the same strain studied by Winslow and Fleeson (1925).

4. Streptococcus pyogenes. A long-chained non-hemolytic organism, originally isolated from pus, courteously provided by Professor L. F. Rettger of the Department of Bacteriology of the Yale Graduate School.

5. Salt-tolerant organism. Salt-tolerant organism no. 4 obtained from Dr. W. S. Sturges of the Cudahy Packing Company laboratories. This organism (a large rod about 2 microns long

by 1 micron wide) is characterized by its power to grow in the presence of 2 M NaCl; but it was easily cultivated on either plain or glucose agar at 37°C. It was thought that the power of this organism to resist the toxic effect of salt would make its reactions of interest.

6. Clostridium flabelliferum. This is the "fish-tail" anaerobe described by Sturges (1926) and was obtained from his laboratory at the Cudahy Packing Company. It was selected, after considerable experimentation with other species, as a representative of the group of obligate anaerobes which, although a spore former, forms spores so slowly that it was easy to obtain cultures containing only vegetative cells. It is a large rod and was cultivated by anaerobic methods (in presence of pyrogallic acid and caustic potash on glucose agar at 37°C.)

7. Mycobacterium smegmatis. This organism was used as a type of the acid-fast group. It was obtained from the laboratories of the New York State Department of Health, Albany, N. Y. Satisfactory growth was made on one per cent glucose agar at 37° in from four to ten days.

8. Bacillus cereus. The same strain of aerobic spore former used in previous studies from this laboratory (Winslow, Falk and Caulfield, 1923; Winslow and Shaughnessy, 1924.) Five-hour cultures at 37°, or eighteen-hour cultures at room temperature, on plain nutrient agar, were found to be almost entirely composed of vegetative cells.

9. Pseudomonas pyocyanea. Obtained from Prof. G. H. Smith of the Department of Pathology and Bacteriology of the Yale Medical School. This organism was of course Gram-negative and had the smallest cells of any type studied, a fact which made it very hard to observe. It was grown on plain nutrient agar for twenty-four hours at 37°C.

10. Bacterium coli I. A strain isolated by Cohen in 1922 and used in previous work from this laboratory (Winslow and Shaughnessy, 1924; Winslow and Fleeson, 1925). These organisms were close to the limit of vision and appeared to grow smaller as the experiments proceeded and results are therefore less accurate than those obtained with larger forms. The organism was grown on plain nutrient agar for twenty to thirty hours at 37°C.

11. Bact. coli II. A second strain, isolated from sewage in this laboratory in 1924.

12. Lactobacillus acidophilus. Several different strains of this organism were used, but, as no significant differences were observed, the results have been grouped together. All were obtained from Prof. L. F. Rettger of the Department of Bacteriology in the Yale Graduate School. They were cultivated for forty-eight hours at 37°C. on one per cent galactose tryptic-digest agar. In preparing this medium 100 cc. "Klim" in 1000 cc. of 1 per cent sodium carbonate solution was allowed to digest with Fairchild's Trypsin powder for two days at 37°C. After adjusting to pH 6.5, 100 cc. of the digest were added to 1 liter of water containing 5 grams of beef extract. Stock cultures were maintained in milk.

13. Capsulated streptococcus. A short chained non-hemolytic Gram-positive bile-insoluble streptococci with well-developed capsules. It was a stock culture courteously furnished by Prof. F. P. Gorham of Brown University. This organism agglutinates spontaneously in solutions on the acid side of the neutral point.

TECHNIQUE OF THE EXPERIMENTS

The organisms to be used in an experiment were in all cases cultivated on a solid agar medium in Kolle flasks or large bottles—the constituents of the agar and the time and temperature of incubation for each species being as indicated in preceding paragraphs. After the proper period of incubation, the cells were washed off in distilled water (or in Ringer's solution or 0.145 M NaCl as the case might be) and then centrifuged and re-suspended in the appropriate menstruum three times. Before making the final suspension the organisms were shaken up with glass beads in a small amount of menstruum and filtered through cotton to remove large clumps or flakes of agar. The final suspension was of such a density as to appear slightly cloudy to the naked eye and to give a few organisms in each microscopic field.

Readings of migration velocity were first made at the initial pH of the suspension, which was about pH 5.4 in water and NaCl

solutions, and about pH 8.0 in Ringer's solution. Adjustment was then made to the various other pH values desired by addition of HCl or NaOH. The pH thus obtained was of course more or less unstable, particularly near the neutral point. The actual pH of the suspension leaving the electrophoretic cell was therefore determined and this value taken as corresponding to the recorded velocities. Readings of pH were made by comparison with the color plates of Clark (1922) while above pH 9.8 the alkaline indicators of Prideaux (1917) were used; although the buffer standards for these indicators proved highly unsatisfactory, on account of their rapid deterioration.

The apparatus used for all determinations was the microscopic cell devised by Northrop (1922) and used with slight modifications by Winslow and Fleeson (1925). A detailed description may be found in the reports of these investigators and need not be repeated here. Suffice it to say that the suspension was run through a glass chamber 1 cm. wide, 7 cm. long and 1 mm. deep, connected at each end with zinc-zinc-sulphate electrodes. Every precaution was taken to secure the highest degree of cleanliness in the apparatus and the cell was freed from air bubbles and thoroughly flushed with each particular menstruum studied before a set of observations was made. A direct current of 112 volts and 12 milliamperes was applied through the non-polarizable electrodes, giving a fall in potential within the cell of about 13 volts per centimeter.

Observations were made with direct illumination and a magnification of 525 diameters. Two different ocular micrometers were used, bearing rulings respectively 30 and 44 micra apart. The rate of migration of five different cells was recorded by the use of a stop-watch at each level observed and the average of the five readings taken as the velocity at that level. The apparatus was always carefully levelled and any possible error due to drifting detected by frequent reversal of the current. In the highly alkaline solutions this was an important precaution since leakage not infrequently occurred as a result of the action of the alkali on the DeKhotinsky cement with which the parts of the cell were joined.

A most important problem to be considered in work of this kind is the level at which observations are to be made. As a result of the phenomena of electrical endosmose, the potential difference between water and glass causes a streaming of the water toward the cathode near the upper and lower glass surfaces of the cell, balanced by a streaming toward the anode in its middle depths. Thus the true velocity of migration of negatively-charged particles will be accelerated at mid-depth and retarded or reversed in the upper and lower levels of the cell. In previous work it has been usual to average readings taken at the mid-points of the three lower sixths or eighths of the cell. For reasons discussed by Winslow and Fleeson (1925) we have concluded that it is more accurate to average readings taken at the mid-points of the upper and lower halves of the cell. The value recorded for any given menstruum in any given experiment was therefore based on the migration velocity of at least 10 different particles, 5 in the upper half and 5 in the lower half of the cell. As a rule four or five experiments were conducted with each organism in each different menstruum, so that the average values given in the tables represent 40 or 50 recorded velocities. In the few cases where only one experiment was made at a given pH the value in the tables is enclosed in brackets and these values have been omitted from the charts.

Throughout this work results have been expressed in terms of velocity in micra per second since as Winslow, Falk and Caulfield (1923) have pointed out there is some possibility of error in the assumption of the constants of the Helmholtz-Lamb equation, by which such velocities are converted into terms of charge upon the migrating particles. Curiously enough, however, the dimensions of the cells and the current-strength used were such that, if the usual assumptions as to dielectric constant and other factors be made (see Winslow, Falk and Caulfield, 1923) the observed migration velocities correspond directly and quantitatively to millivolts of potential difference between suspended particles and menstruum.

PRESENTATION OF DETAILED RESULTS

The average migration velocities observed at each pH value in each of the three menstrua studied are presented below in table 1 (water), table 2 (Ringer's solution) and table 3 (0.145 M NaCl); and the results are presented graphically in figures 1 to 3. In all charts, ordinates represent velocities in micra per second, and abscissae represent pH values.

GENERAL CHARACTERISTICS OF ELECTROPHORETIC MIGRATION AS INDICATED BY OUR RESULTS

It will be evident from a survey of tables 1 to 3 and figures 1 to 3 that our results, so far as the general phenomena of electrophoretic migration are concerned, are in complete harmony with those of previous workers in this field. We find that all of the suspended particles studied, whether organic or inorganic, living or dead (the microörganisms must of course have been killed by the more extreme acidities and alkalinities employed) bear a negative charge at ranges of hydrogen ion concentration above pH 2.0.

This charge reaches a maximum at two points on either side of neutrality. The exact relation of the two maxima, and the slight dip between them, varies somewhat, as would be expected, since the reaction of an unbuffered solution near the neutral point is so difficult to adjust with accuracy, *B. cereus* and *Bact. coli* I show a smooth curve with a maximum at neutrality while *St. pyogenes* shows a peak on the alkaline side and *Bact. coli* II a peak on the acid side of neutrality. For *Ps. pyocyanea* there are too few data to make our results significant. In all other cases (*L. acidophilus*, *Cl. flabelliferum*, salt-tolerant organism, *Sacch. apiculatus*, *Chlorella*, *Myco. smegmatis*, the capsulated streptococcus and silica dust) we find a fairly high velocity at neutrality which rises to a maximum on either side of the neutral point. The same phenomena are shown by the data of Winslow, Falk and Caulfield (1923) and Winslow and Fleeson (1925). We believe it to be a general one and are inclined to assume that

TABLE 1

Average migration velocities (in micra per second) of various vegetable microörganisms and silica dust particles at various pH values

Suspended in distilled water

	pH											
	1.5	2 5	3 5	4 5	5.5	6 5	7.5	8 5	9 5	10 5	11.5	12.5
Silica dust	−10.8	−14.7	−16.2	−15.8	−13 8	−14.0	−15.0	−21.5	−22.1			−19.1
Chlorella	−1.5	−4.0	−13.8	−14.4	−19.8	−23.1	−17.5	−21.6	−18.1			−13.5
Sacch. apiculatus	−4.1	−5.4	−7.6	−7.7	−20.3	−19 9	−17.1	−15.0	−17.2		−10.0	−5.9
St. pyogenes	−0.9	−2.9	−9.4	−14.1	−17.2	−17.7	−18.7	−24.5	−20.4	−20.0	−15.8	−9.7
Salt-tolerant organism	−0.3	−0.2	−11.1	−13.4	−15 5	(−22 1)	−17.2	(−19.3)	−18 6			−9.9
Cl. flabelliferum	+1.1	+1.1	−9 5	−9.8	−19.6		−15.1	(−17.9)	−23.2			−17.6
Myco. smegmatis	+3.0	+4.3	−3.5	−13.0	−23.5	−19.0	−20.4	−26.3	−22.4	−22.2	−14.9	−9.3
B. cereus	+1.7	+4.7	−1.0	−12.9	−14 0	−15.4	−19.6	−18.3	−18.5		(−10.3)	−11.2
Ps. pyocyanea	(+2.9)	(+1.9)	(−13.0)		(−15.0)	(−15.0)		(−27.0)				(−10.0)
Bact. coli I	0	−1.9	−6 0	−12.4	−11.8	−14.5	−14.9	−14.1	−11.9	(−5.1)	−7.3	−5.1
Bact. coli II	+1.4	0	−9.5	−8.4	−13 2	(−20.0)	−15.0	(−9.4)	−10.8			−8 9
L. acidophilus	+2.3	+2 4	−4.0	−12.1	−14 6	−17 2	−13.4	−18.8	−14.8	−17.0	−15.0	−10.3
Capsulated streptococcus	+2.1	+0 1	−1.4	−11 3	−6 1	−8.2	−9.7	−12.9	−9.3			−9 5

\+ indicates migration toward cathode; − indicates migration toward anode.
Figures in parentheses based on a single experiment only and omitted from the charts.

TABLE 2

Average migration velocities (in micra per second) of various vegetable microörganisms and silica dust particles at various pH values

Suspended in Ringer's solution

	pH											
	1.5	2.5	3.5	4.5	5 5	6.5	7.5	8.5	9 5	10.5	11.5	12.5
Silica dust	−9.1	−10.0	−10.4	−14.0	−14.6	−17.1	−16.5	−14.7	−13.4			−10.9
Chlorella	−7.5	−9.7	−12.3	−10.7	−10 4	−12.7	−12.5	−13.0	−12.3			−12.7
Sacch. apiculatus	−5.8	−5.8	−6.8	−7.8	−8.2	−7.5	(−7.4)	−9.2	−8.4		−9.4	−8.2
St. pyogenes	−5.7	−6 2	−7.2	−8 6	−9.2	−9 7	−9 8	−10.6	−11.2	−10.7	−10 0	−9.9
Salt-tolerant organism	−6 6	−7.2	−10 4	(−9 3)	−12 0	−13 6	−12.4	−14.1	(−14.4)			−13.2
Cl. flabelliferum	−0.8	−1 7	−3 7	(−9 7)	−9 3	−10 5	−11.5	−11.3	−13 8			−12.0
Myco. smegmatis	+1 4	+1 4	−2 9	−4 0	−11.3	−10.3	−12.3	−9 9	−14 5			−10.0
B. cereus	−5 7	−6 2	−6 8	−7 7	−8.6	−9 5	−7 8	(−11.1)	−8.5			−9.7
Ps. pyocyanea	0	0	−4 5	−6.2		−8 6		−7.1	−7.1			−8.7
Bact. coli I	−0 5	−1 0	−2 2	−3.8	−3.2	−4 9	−5.7	−5 2	−4.9		−7.9	−5.1
Bact. coli II	(−2 8)		(−4.3)	−4.7	(−5.9)	(−6.6)		−5.7				(−6 8)
L. acidophilus	+0 6	+0 1	−0 9	−4.3	−4.5	−5.8	−5.4	−7.2	−8.5	−6.9		−7.4
Capsulated streptococcus	−1 5	−3 7	0 0	−2 4	−6.2	−3 5	−6 1	−4.8	−6 1			−5 9

+ indicates migration toward cathode; − indicates migration toward anode.
Figures in parentheses based on a single experiment only and omitted from the charts.

TABLE 3

Average migration velocities (in micra per second) of various vegetable microörganisms and silica dust particles at various pH values

Suspended in 0.145 M NaCl

	pH											
	1.5	2.5	3.5	4.5	5.5	6.5	7.5	8.5	9.5	10 5	11.5	12.5
Silica dust	−10.1	−12.6	−14.5	−13.9	−14.3	−17.0	−20.1	−16.0	−18.0			−16.4
Chlorella	−7.2	−6.2	−7.7	(−8.3)	−11.4	−11.8	−12.3	−10.4	−11.3			−12.5
Sacch. apiculatus	−4.9	−4.9	−5.6	−7.1	−8.0	−6.3	(−12.5)	−7.8	−8.3			−9.1
Salt-tolerant organism	−5.0	−7.3	−9.5		−14.1	−13.1	(−14.6)	−15.0				−13.0
Myco. smegmatis	−3.9	−4.0	−7.6	(−12.1)	−12.1	(−12.7)	−16.2	(−10.7)	(−15.0)			−12.8
B. cereus	−7.2	−7.8	−9.9	−10.3	−10.8	−12.0	−12.4	−12.4	(−11.3)			−10.4

\+ indicates migration toward cathode; − indicates migration toward anode.
Figures in parentheses based on a single experiment only and omitted from the charts.

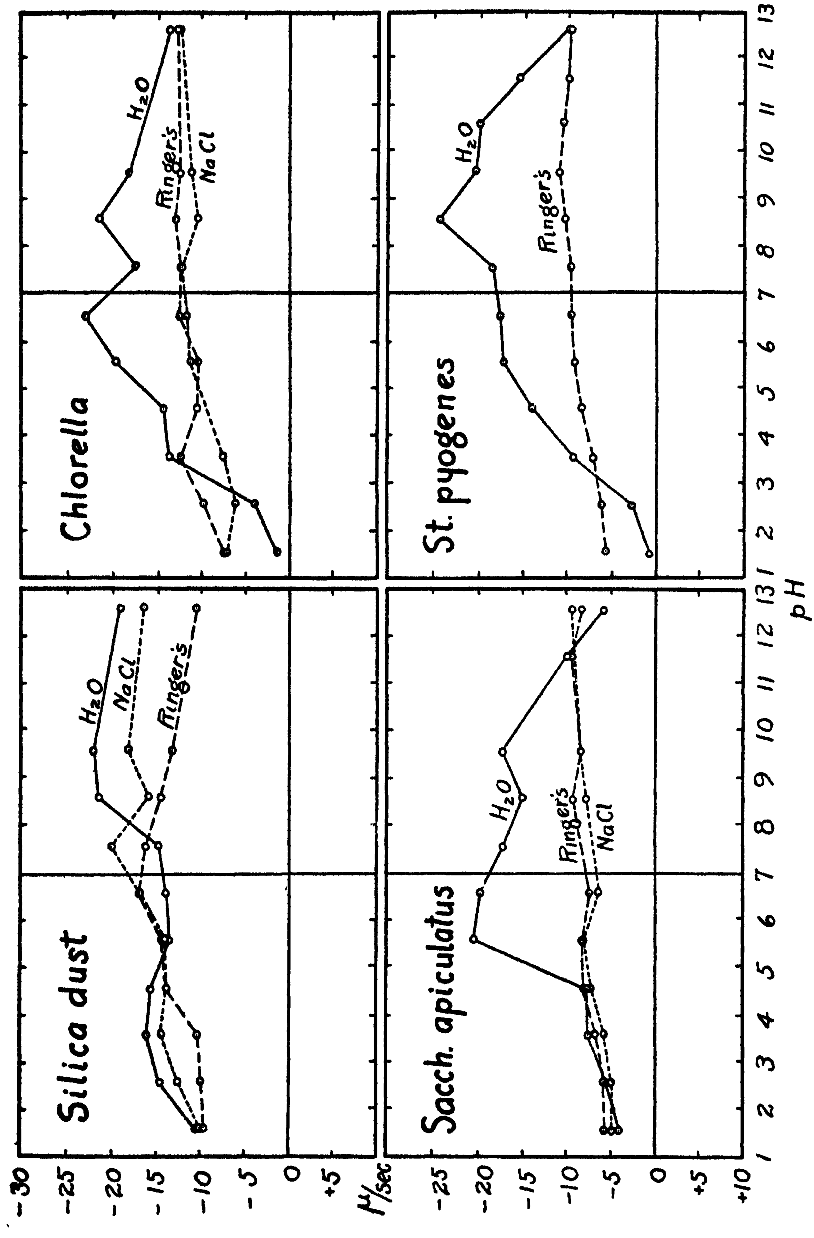
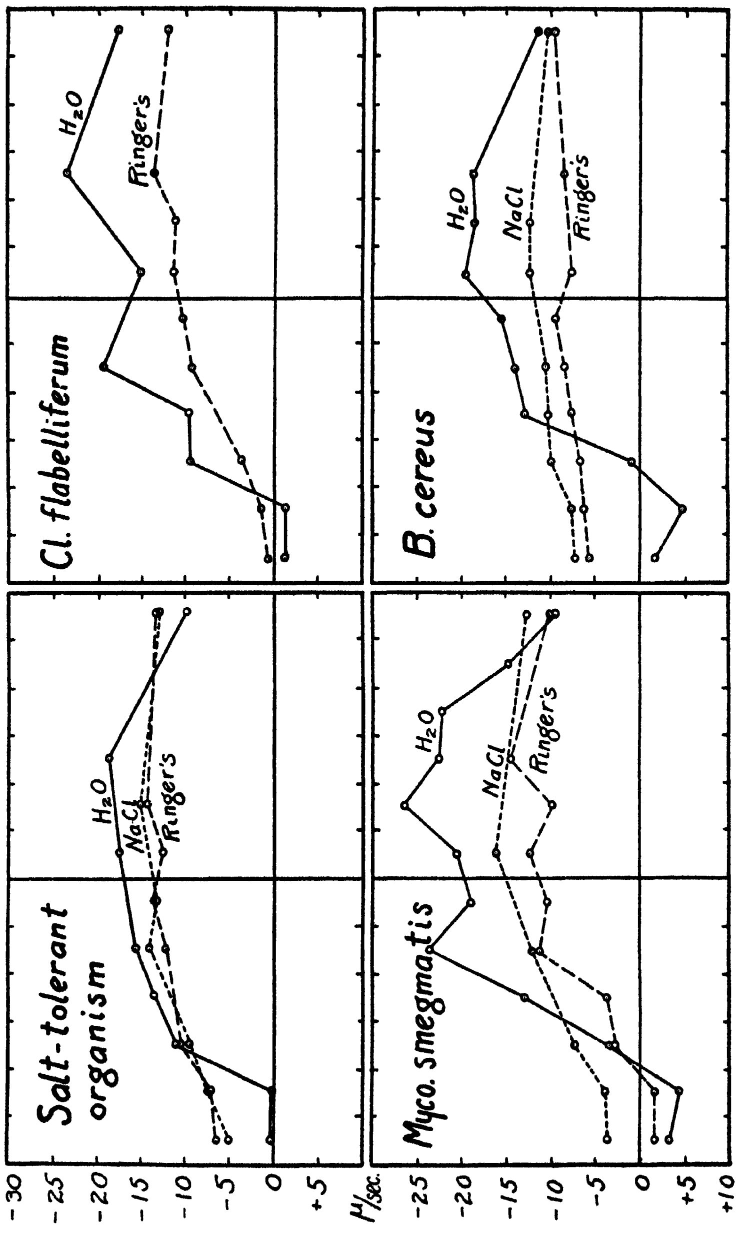

Silica dust
Chlorella
Sacch. apiculatus
St. pyogenes
H₂O
NaCl
Ringer's
μ/sec
pH

FIG. 1



Salt-tolerant organism
Cl. flabelliferum
Myco. smegmatis
B. cereus
H_2O
NaCl
Ringer's
pH
μ/sec.
-30 -25 -20 -15 -10 -5 0 +5
-25 -20 -15 -10 -5 0 +5 +10
1 2 3 4 5 6 7 8 9 10 11 12 13

FIG. 2

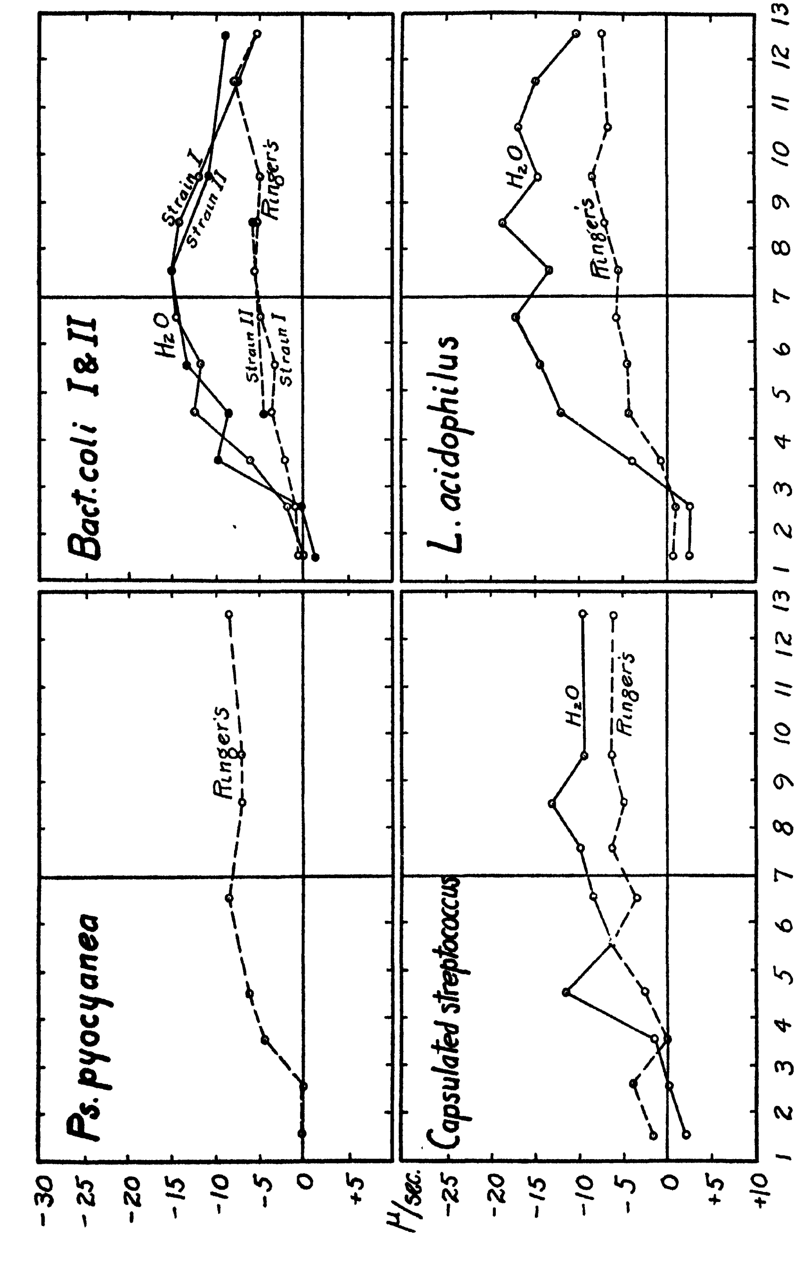

Ps. pyocyanea
Ringer's
Bact. coli I & II
H2O
Strain I
Strain II
Ringer's
Strain II
Strain I
Capsulated streptococcus
H2O
Ringer's
L. acidophilus
H2O
Ringer's
μ/sec.
-30 -25 -20 -15 -10 -5 0 +5
-25 -20 -15 -10 -5 0 +5 +10
1 2 3 4 5 6 7 8 9 10 11 12 13
1 2 3 4 5 6 7 8 9 10 11 12 13
pH

FIG. 3

where a depression in the curve at the neutral point is not recorded, it is because the actual neutral point was missed in making the tests.

The further addition of acid or alkali (beyond the maxima which occur near pH 6.5 and pH 8.5) leads to a progressive decrease in charge, which is much more rapid on the acid than on the alkaline side. With the pH values studied by us (extending only to pH 12.5 on the alkaline side) the velocity of the particles decreased in alkaline solution only to between 5 and 14 micra while *Cl. flabelliferum* and the silica dust showed even less effect. On the acid side, however, the velocity of even the silica dust was reduced to less than 11 micra at pH 1.5 while none of the microörganisms studied had a velocity as high as 5 micra and seven of them showed a reversal of charge, at this hydrogen ion concentration.

The effect of Ringer's solution and of 0.145 M NaCl is similar to that of acid or alkali. In both cases the charge at all pH values near neutrality is markedly reduced. The maximum velocity for silica dust observed in Ringer's solution was about 17 and the maximum for any microörganism was 14.5 micra. On the alkaline side, velocities in these salt solutions are not appreciably affected by change in reaction, remaining in general at about 9 micra from pH 8.5 to 12.5. On the acid side, however, the velocity falls with additional acidity, but not nearly so rapidly as is the case in distilled water. Beyond pH 3.5 the curves for water and salts generally cross each other, the velocity of migration in Ringer's solution at a given pH being greater than in water of the same reaction. This is manifest in the case of every organism studied but was not shown by the silica dust. The phenomenon was observed by Winslow, Falk and Caulfield (1923) and is, we believe, a general one. Its significance will be discussed in a succeeding paragraph.

While, as will later be emphasized, there are distinct and important differences between the effects of electrolytes upon the various microörganisms studied, their reactions are sufficiently alike to make it seem worth while to average the results obtained with different species so as to present a rough composite

TABLE 4

Average velocities of all microörganisms studied in each menstruum at various pH values

		pH											
		1 5	2.5	3 5	4 5	5.5	6.5	7.5	8 5	9 5	10 5	11.5	12.5
5 organisms*	0.145 M NaCl	–5.6	–6.0	–7.9	–9 7	–11 3	–11.1	–13.6	–11.3	–11.5			–11.6
	Ringer's	–4 8	–5 5	–7.8	–7.9	–10.1	–10.7	–10 5	–11.5	–11 6			–10.8
11 organisms†	Ringer's	–3.1	–3.6	–5 6	–7 0	–8.3	–9.1	–9.4	–9.5	–10.4	–8.8	–9.1	–9.4
	Water	+0.5	0.0	–7.1	–11.8	–16.8	–18.4	–16.9	–19.3	–17.7	–16.1	–10.5	–10.1

* Chlorella, salt-tolerant organism, *Sacch. apiculatus*, *Myco. smegmatis*, *B. cereus*.

† Five organisms listed under (1) plus *St. pyogenes*, *Cl. flabelliferum*, *Ps. pyocyanea*, *Bact. coli* I, *Bact. coli* II and *L. acidophilus*.

picture of the group as a whole. This has been done in table 4, comparing in the first two lines the values obtained in NaCl and Ringer's solution for the five species studied in the former menstruum and in the last two lines the values obtained in Ringer's solution and in water for all the organisms studied except the capsule-bearing form. This capsulated organism and the silica dust are so different from the rest in their behavior that they have been omitted from the calculation.

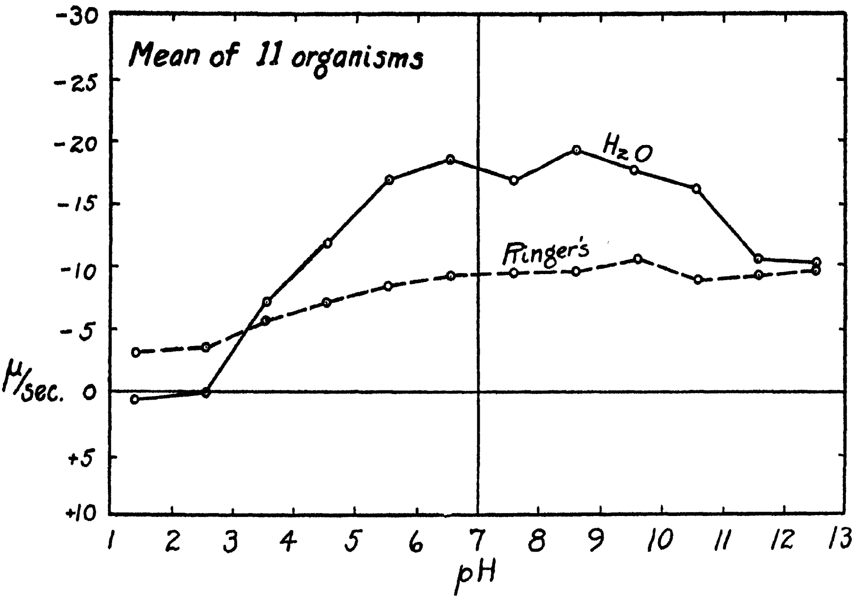


FIG. 4

The results of this composite average, as presented in figure 4, give a very clear picture of the general phenomena observed. We note a migration velocity of about 17 at pH 7.5 rising to 18 at pH 6.5 and to 19 at pH 8.5. On the alkaline side, the composite curve falls with increasing alkalinity to 10 at pH 12.5. On the acid side it falls more rapidly to an isopotential point at pH 2.5 and the charge becomes positive at pH 1.5. In Ringer's solution the composite curve remains between 8 and 10 at all reactions between pH 7.5 and pH 12.5. On the acid side the

velocity falls slowly with increasing acidity to 3 at pH 1.5 but is higher than that observed in corresponding pH ranges in distilled water beyond pH 3.5.

VARIATIONS IN ELECTROPHORETIC MIGRATION CHARACTERISTIC OF VARIOUS TYPES OF SUSPENDED PARTICLES

We may now turn from the general characteristics of the electrophoretic curve to the specific peculiarities of response exhibited by different types of vegetable microörganisms. Reference to tables 1 to 3 and figures 1 to 3 will make it at once clear that such differences actually exist.

First of all, it is evident that the velocity of migration of silica dust is much less influenced by either acid or alkali or salts than in the case of the organic cells here studied. Itsmaximum velocity of 22.1 in water is reduced only to 10.8 at pH 1.5 and to 19.1 at pH 12.5.—in Ringer's solution at pH 6.5 only to 17.1. The migration velocity of *Chlorella* is more affected by either acid or salt than is that of silica dust and *St. pyogenes* and *Sacch. apiculatus* come next in order. All these organisms as indicated in figure 5 show curves in Ringer's solution which are fairly flat on the alkaline side and dip gently on the acid side but they lie at slightly different levels.

Myco. smegmatis and *Cl. flabelliferum* show a distinctly different type of curve (fig. 6), at a high level on the alkaline side, but falling off sharply on the acid side. These organisms are characterized by a moderate effect of salt (similar to that shown in the *Chlorella* group) but a very marked effect of acid. *B. cereus* and the salt-tolerant organism are probably to be classed in this group, since their curves in water fall very low in the acid range although for some reason they do not dip steeply at the acid end of the Ringer's curve.

A third group (fig. 7) includes *Ps. pyocyanea*, the two strains of *Bact. coli* and *L. acidophilus* which are very markedly affected by both acid and salts, showing a curve in shape like that of Group 1 but at a much lower level. Finally, the capsulated streptococcus differs from all the other organisms studied in its low migration velocity at all pH values. The highest recorded

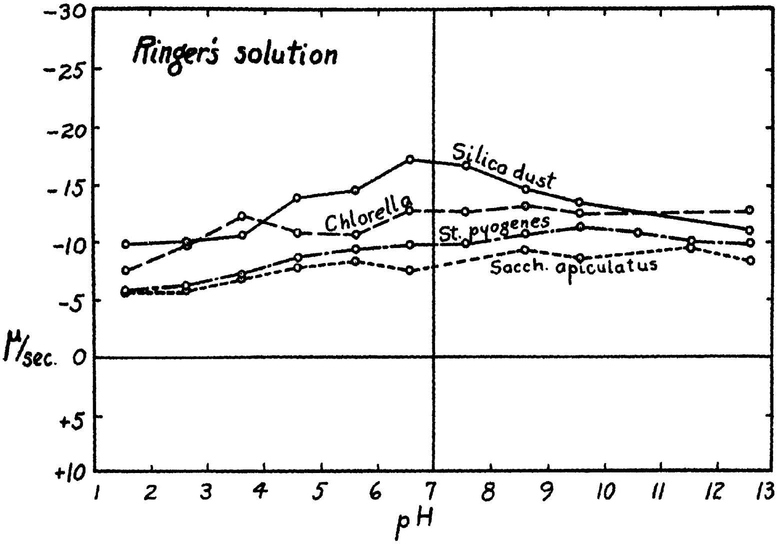

Ringer's solution
Silica dust
Chlorella
St. pyogenes
Sacch. apiculatus
μ/sec.
pH
-30
-25
-20
-15
-10
-5
0
+5
+10
1 2 3 4 5 6 7 8 9 10 11 12 13

FIG. 5

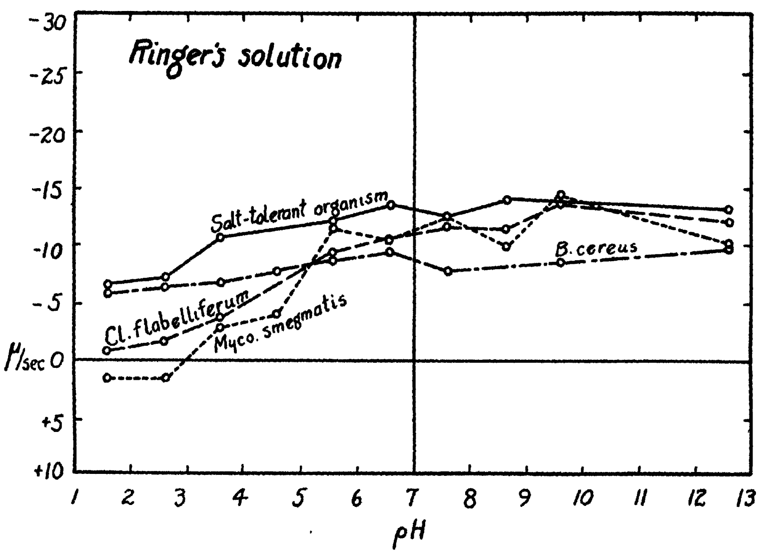
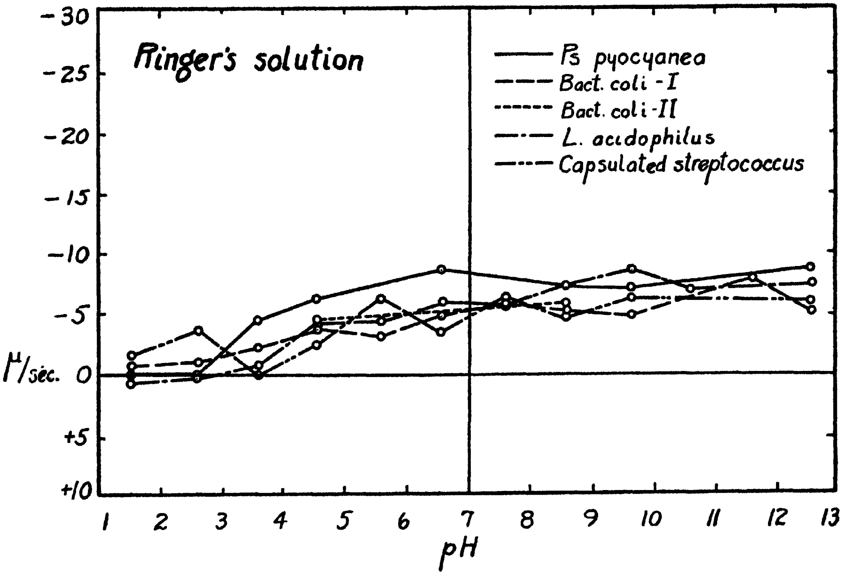
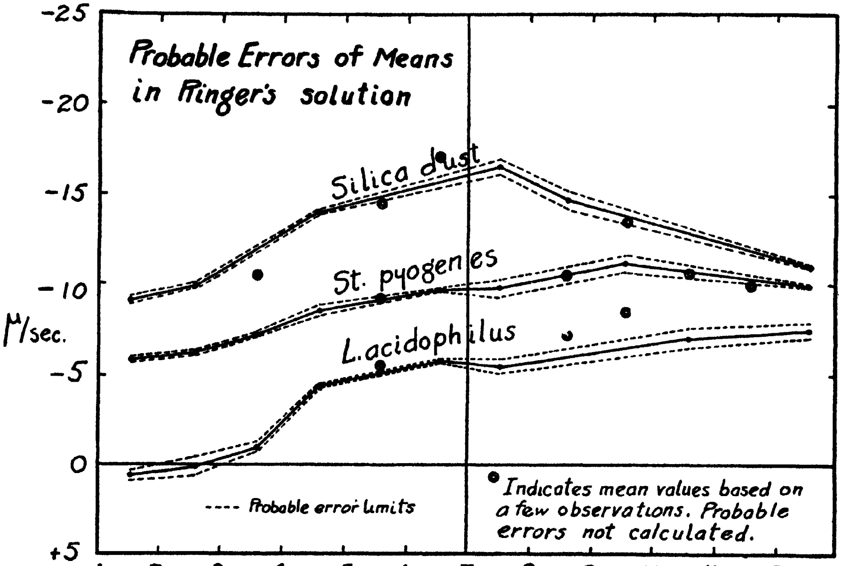

Ringer's solution
Salt-tolerant organism
B. cereus
Cl. flabelliferum
Myco. smegmatis
μ/sec
pH
-30
-25
-20
-15
-10
-5
0
+5
+10
1 2 3 4 5 6 7 8 9 10 11 12 13

FIG. 6



Ringer's solution
Ps pyocyanea
Bact. coli -I
Bact. coli -II
L. acidophilus
Capsulated streptococcus
μ/sec.
pH

FIG. 7



Probable Errors of Means in Ringer's solution
Silica dust
St. pyogenes
L. acidophilus
μ/sec.
---- Probable error limits
• Indicates mean values based on a few observations. Probable errors not calculated.
pH

FIG. 8

velocity in water was 12.9 micra at pH 8.5. In Ringer's solution its curve is like that of the other organisms plotted in figure 7, lying at a low level throughout.

In considering such results as those here presented, a question naturally arises as to the extent to which the figures are reliable and the consequent weight which may be placed upon any specific differences observed. Marked variations between duplicate observations made at different times do, no doubt, occur as is almost always the case when biological reactions are studied in detail. In order, to see whether the differences between the main groups were really significant we have computed for three typical curves (see figure 8) the probable error of the mean velocities at various pH value in Ringer's solution. (The large dots represent values based on but one or two observations for which of course no probable errors could be calculated.) The bands between the dotted lines enveloping each curve represent the limits of the probable error, as computed by the usual formula from our individual observations; and it will be noted that the band for silica dust remains at all points well above that for *St. pyogenes* and that for *St. pyogenes* well above that for *L. acidophilus*.

TABLE 5

Migration velocities of five different strains of non-capsulated streptococci

STRAIN	WATER			RINGER'S SOLUTION		
	pH 2.5	pH 5 5	pH 9.5	pH 2.5	pH 5.5	pH 9 5
1	−2.9	−17.2	−20.4	−6.2	−9.2	−11.2
2	0	−15.7	−20 8	−0.8	−6.0	−6.4
3	−5.8	−20.5	−20.8	−5.9	−7.3	−9 1
4	−8.1	−16.2	−15.6	−7.7	−10.5	−12 2
6	−3.4	−22 9	−16.4			

It is significant to observe that the curves for the two strains of *Bact. coli* are essentially the same. No. I shows a tendency to a slightly higher charge than no. II but the curves evidently belong to the same general type. To test further the constancy of reaction of different organisms belonging to the same general group we made a special study of four additional strains of non-capsulated streptococci at certain typical pH ranges for com-

parison with our first strains of *St. pyogenes.* The results are presented in table 5.

Strain no. 1 (our primary strain) was long-chained non-hemolytic and did not produce green coloration on blood agar; strains 2 and 3 were both of the *Str. viridans* type from blood cultures; strain 4 was a non-hemolytic type from urine; strain 6 was a hemolytic strain. All of these five organisms were essentially similar in their reactions although no. 2 showed a slightly lower charge than the others.

SUMMARY OF CONCLUSIONS AND DISCUSSION OF RESULTS

It seems evident from our work, as well as from that of the other observers who have been cited, that vegetable microörganisms, like silica dust particles and collodion particles, normally bear a considerable negative charge, which causes them to migrate toward the anode in an electrical field. If the usual assumptions be made as to dielectric constant, etc., this migration velocity corresponds to a charge of about 17 millivolts.

Slight additions of acid (pH 6.5) or alkali (pH 8.5) or of neutral salts, cause an increase in velocity corresponding to a charge of 18 to 20 millivolts.

Further addition of electrolytes causes a decrease in charge, which is dependent on the concentration of cations (NaOH and NaCl producing identical effects as shown by Winslow and Fleeson). As would be expected this effect of the cations is proportional to their valency but the hydrogen ion exhibits a special power due to its high migration velocity. With hydrogen and with trivalent ions the charge on the cells is frequently entirely reversed and becomes positive. This does not occur with ordinary concentrations of univalent cations but may be noted in very extreme concentration (in alkali at pH 14.0 as shown by Winslow and Shaughnessy, 1924).

In a mixture of cations other than hydrogen the resultant effect is generally cumulative. With a mixture of hydrogen and other univalent cations on the other hand (such as acid Ringer's solution) a very interesting effect is observed. The influence of the hydrogen ion is to a considerable extent neutralized by the

ions of less potency. We are inclined to believe that in some fashion not clearly understood the sodium ions replace hydrogen ions in union with the cell or in its electrical double layer and thus produce an effect analogous to that of a buffer, although there is of course no buffer effect in the ordinary sense, since the pH value of the more effective water and the less effective salt solution is identical.

While these general effects are always manifest, distinct and significant differences may be observed between electrophoretic migration curves plotted for various types of suspended particles. Silica dust is much less markedly reduced in charge than are the vegetable cells, by either hydrogen or other cations. *Chlorella* is least affected of the organisms studied. The streptococci and *Sacch. apiculatus* are somewhat more strikingly reduced in charge while colon bacilli *Ps. pyocyanea* and *L. acidophilus* show an even greater reduction in velocity. *Myco. smegmatis* and *Cl. flabelliferum* are only moderately affected by salts (yielding a curve in the alkaline range like that of *Chlorella*) but are markedly affected by hydrogen, falling off very sharply in the acid range. *B. cereus* and the salt-tolerant organism are somewhat aberrant, showing only a moderate fall in charge with increased acidity in Ringer's solution but dropping rapidly in water in the presence of acid.

A capsulated streptococcus studied shows a marked difference from any other type in its extremely low velocity of migration. The reasons for these differences can only be conjectured. There is apparently no relation to the Gram reaction such as has been postulated by Stearn and Stearn (1925), since *Bact. coli* and *L. acidophilus* show identical results. It is perhaps significant that the yeast and the alga which were the largest organisms studied showed less effect of electrolytes than did the bacterial cells and that the capsulated organisms (perhaps on account of the chemical nature of their capsule) showed a very low velocity under all conditions. The differences observed seem in any case to be reasonably constant and significant and to indicate that the chemical and physical constitution of the cell plays at least some part in determining the effect of electrolytes upon electrophoretic charge.

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SURFACE TENSION AND BACTERIAL GROWTH[1]

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HISTORICAL RÉSUMÉ

We have found only a few reports which bear directly on surface tension as a factor in bacterial growth.

Larson, Cantwell, and Hartzell (1919), studied the effect of lowered surface tension on the growth and characteristics of certain organisms, and concluded that the growth of bacteria in ordinary broth was greatly influenced by the surface tension of the medium. The surface tension was lowered by additions of castor oil soap and measured by the drop weight method. The standard broth which was employed had a surface tension of 59 dynes per square centimeter. *B. subtilis* did not form a pellicle when the surface tension was reduced to 45 dynes, but grew down in the body of the medium. They concluded that all pellicle formers ceased to grow at the surface when the surface tension was below 45 dynes per centimeter. *B. subtilis* was found to form spores more slowly in a medium of lowered surface tension.

Some anaerobes, particularly *B. tetani*, were found to grow aerobically in a medium of lowered surface tension. The authors suggest that the favorable action of the oil seal in anaerobiosis is probably due to the lowered surface tension. They are inclined to believe that the toxicity of the depressant is a factor of more or less importance. In a later report Larson (1921) found the surface tension of ordinary broth to be about

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59 dynes, and of inorganic media with glycerol as a source of carbon, 73 dynes. The surface tension was depressed by additions of castor oil soap, giving a range from 32 to 73 dynes per square centimeter. The growth of pneumococcus and streptococcus was depressed at a surface tension of 50 dynes and lower. The limit of *B. anthracis* was sharply defined at 46 dynes. The intestinal bacteria grew well at lowered surface tension. The ability of this group to develop in media of low surface tension was thought to be related to the resistance of the group to bile, which has a low surface tension. The concentration of the colon-typhoid group of bacteria at the surface of the medium was attributed to the concentration of the surface tension depressants at the surface. Pellicle formers were found to grow throughout the body of the medium when the surface tension was reduced, while non-pellicle formers were found to develop pellicles after long cultivation in media of high surface tension. Symbiosis and antibiosis, were, in some cases, attributed to surface tension.

Ayers, Rupp, and Johnson (1923) studied the effect of surface tension on the streptococci. Castor oil soap, sodium glycocholate, iso-amyl alcohol, and sodium oleate were used as depressants. The basic medium employed had a surface tension of 59.6 dynes, and was depressed as low as 40 dynes. These authors conclude that some species of streptococci are retarded in growth when the surface tension of the medium is lowered to 53 dynes, and suppressed at 45 dynes, while others are retarded at 43 dynes. Still other species are depressed at 40 to 41 dynes. In general 40 dynes prevented growth. *Streptococcus pyogenes* was the most susceptible to lowered surface tension, and *Strept. lactis* least affected. Some toxicity of the depressants was noted.

Mellon, Hastings, and Anastasia (1924) studied the cohesive power responsible for the spontaneous agglutinability of certain bacteria. They found that this power could be diminished or entirely eliminated by varying the surface tension of the solution. Strains which immediately agglutinated, or failed to produce emulsions, were found to emulsify readily in a solution contain-

ing sodium oleate. This was attributed to the interfacial tension between organism and solution.

EXPERIMENTAL METHODS

Preparation of broth. The stock broth used in the experiments was prepared as follows: Parke Davis and Company, pepton 10 grams, beef extract 3 grams, distilled water 1000 grams. These ingredients were cooked together in the steamer one and one-half hours, the reaction adjusted to pH 7.0 and steamed for another half hour, then cooled and filtered under pressure through macerated filter paper. Broth prepared in this manner had a surface tension of 48 to 50 dynes per square centimeter.

Surface tension depressants. Soaps were prepared from castor oil, cocoanut oil, olive oil, and palmitic acid. The oils were saponified by adding an alcoholic solution of KOH in excess and heating under a reflux condenser for several hours, then allowing to stand overnight. The solution was neutralized to phenolphthalein by a solution of HCL in absolute alcohol and filtered hot through filter paper to remove chlorides. It was then concentrated to small volume and poured into an excess of saturated solution of sodium chloride in water. The precipitated glycerides were collected on filters and washed with saturated sodium chloride solution, then dried at 28°C. and finally dried at 60°C. They were then dissolved in absolute alcohol, filtered, again evaporated and dried, and again taken up in absolute alcohol and filtered. They were then dried at 28°C. and finally at 60°C. and stored for use.

Stock solutions of the four soaps were prepared by dissolving 2 grams in 100 cc. of distilled water. These 2 per cent solutions were then cooled to just above the freezing point and filtered under pressure through macerated filter paper. This cooling and filtering process was repeated until the solutions were perfectly clear and gave little or no precipitate when added to the stock broth. These 2 per cent solutions were used as bases and were added to broth or other media by pipette. A broth referred to as containing 5 per cent soap contains 5 cc. of this 2 per cent soap solution per 100 cc. of medium.

The four soaps mentioned were used throughout the work, in order that any phenomena observed, in reality due to the nature of the depressant, would not be ascribed to surface tension. This precaution was justified in nearly every experiment.

The surface tension depressing efficiency of these soaps varied considerably, not only in the actual depression possible, but in the proportion of soap necessary to produce it. With each soap there was a maximum concentration, beyond which further additions had little effect on the surface tension. With castor soap this was 1 per cent, with olive soap about 2 per cent, with palmitic soap 6 per cent, and with cocoanut soap as high as 10 per cent. From the basis of the amount of soap necessary to produce a given depression, cocoanut soap was the most efficient, 0.1 per cent giving a depression of 7 to 8 dynes and 1 per cent giving a depression of 18 dynes. Olive soap was only slightly less efficient. Palmitic soap was used in concentrations as high as 5 per cent without securing any more depression of the surface tension than with olive or castor soap at 1 per cent. Cocoanut produced the lowest surface tension of any of the soaps but was less satisfactory, due to the fact that it sometimes produced turbidity or precipitates. Castor and palmitic soaps gave perfectly clear solutions in all concentrations used and olive soap slightly opalescent solutions but without precipitates.

The possible toxicity of depressants is an important factor. These four soaps varied considerably in this respect to different organisms. Some organisms were very susceptible to a given soap, others apparently not at all.

Surface tension measurement. The surface tension was determined by the film method as outlined by Fahrenwald (1922). In one experiment, the drop weight method was also employed for the sake of comparison. The film method has the advantage that several determinations can be made in a relatively short time, not at the expense of accuracy. It is also possible by this method to distribute the surface tension depressants, which accumulate at the surface, throughout the body of the medium, thus enabling one to check on separate determinations. It will appear that the surface tension varies slightly with each measure-

ment of the same solution, and an equilibrium cannot be obtained. This may be avoided by gently stirring the depressants into the body of the medium, then quickly measuring the tension. A second measurement will then be found to check the first. Results obtained by the method of Fahrenwald are lower than those obtained by the drop weight method.

EXPERIMENTAL DATA

Escherichia coli and surface tension

Beef extract broth containing the various depressants was inoculated with a twenty-four-hour culture of *Escherichia coli* and incubated at 28°C. The flasks were so arranged that the surface tension could be checked at intervals to determine the effect of the metabolism of the organism on the surface tension of the medium. The pH of the broth was 7.0. Table 1 shows the arrangement of the cultures, the surface tension, and the hydrogen ion concentration. Each soap had a series not inoculated in order to trace changes due to factors other than bacterial development. The surface tension was determined by the method of Fahrenwald and by the drop weight method. The results are shown in table 1 and chart 1.

It is seen from the data in the table and chart that the depressants gave a range in surface tension, as determined by the film method from 41.2 to 51.2 dynes per cubic centimeter. The same broths as determined by the drop weight method gave a range of 54.2 to 63.2 dynes per cubic centimeter. The latter method in this case gives, in general, results about 12 dynes higher than the film method. It will be noted that the difference in the two methods remains somewhat constant throughout. In discussing the results the data obtained by the film method will be taken.

The surface tension in the uninoculated control broth increased 1.5 dynes per cubic centimeter during the ten-day period. The hydrogen ion concentration remained constant. The uninoculated broths which received depressants gave a general small increase in surface tension during the period, an average increase

TABLE 1

Escherichia coli and surface tension

TREATMENT 2 PER CENT SOLUTION PER 100 CC. BROTH	TIME OF INCUBATION	pH	SURFACE TENSION PER CENTIMETER			
			Inoculated.		Not inoculated	
			Film method	Drop weight method	Film method	Drop weight method
	days		*dynes*	*dynes*	*dynes*	*dynes*
None	0	7.0	51.2	63.2	51.2	63.2
	1	7.1	54.3	64 4	53.4	63.9
	2	7.8	52.5	61.9	51.4	61.4
	3	8.0	51.8	62.2	52.4	63.7
	5	8.3	51.9	65.6	52.3	63.5
	7	8.3	52.6	65.6	52.0	64.8
	10	8.3	54.8	70.0	52.7	64.3
Castor oil soap, 0.1 cc	0	7.0	44.9	58.3	44.9	58.3
	1	7.3	48.5	60.8	47.8	58.3
	2	7.7	49.4	59.3	46.6	56.6
	3	8.1	50.0	61.5	47.3	58.7
	5	8.2	51.4	64.1	47.1	59.7
	7	8.4	52.8	65.6	47.2	57.7
	10	8.4	53.4	69.1	47.5	63.5
Olive oil soap, 0.2 cc	0	7.0	46.8	59.6	46.8	59.6
	1	7.0	49.7	62.9	49.1	62.2
	2	7.6	50.3	60 7	49.7	61 4
	3	8.1	49.6	61 5	49 0	60 8
	5	8.2	50.4	64 1	48.7	62.5
	7	8.3	51.4	64.6	49.1	60.3
	10	8.3	51.0	68.2	48.5	63.5
Cocoanut oil soap, 1 cc	0	7.0	41.2	54 2	41.2	54.2
	1	7.2	45.1	56.5	44.3	55.2
	2	7.6	49.1	61.4	43.5	53.8
	3	8.1	49 6	60.8	44.4	54.5
	5	8.3	51.8	64.1	43.2	54.8
	7	8.4	51.6	64.0	44.9	53.6
	10	8.4	52.0	69.1	43.8	58.7
Palmitic soap, 3 cc	0	7.0	43.6	57.7	43.6	57.7
	1	7.1	47.1	60.1	48.3	62.2
	2	7.8	48.1	61.4	47.2	61.4
	3	7.9	47.5	59.4	46.1	57.5
	5	8.1	49.2	62.5	46.1	56.5
	7	8.2	50.1	63.2	46.4	59.0
	10	8.3	53.0	63.5	46.8	61.4

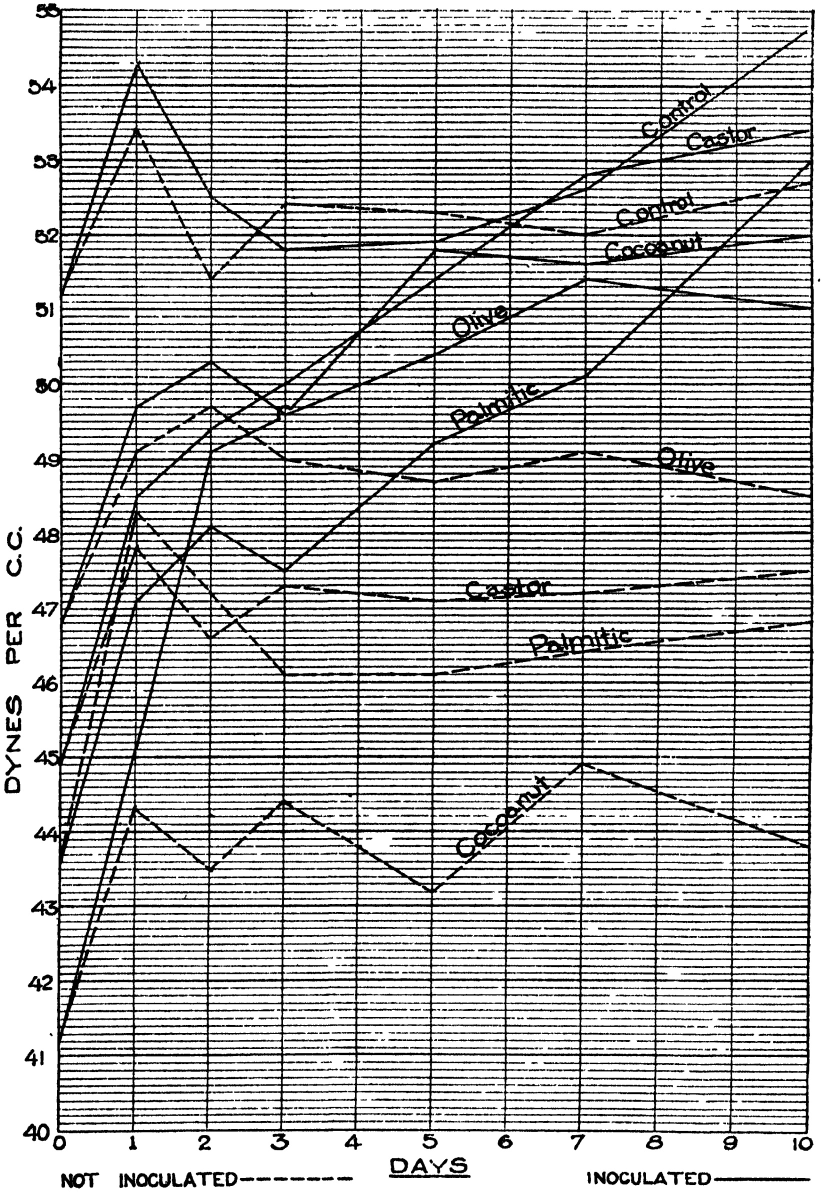

CHART 1. ESCHERICHIA COLI AND SURFACE TENSION

of 2 to 3 dynes, while the hydrogen ion concentration remained constant. The inoculated flasks, with the exception of the untreated control, gave an increase in surface tension varying from 4.2 to 10.8 dynes per cubic centimeter. The control inoculated flask increased but 3.6 dynes per cubic centimeter in the ten days. The maximum increase in surface tension occurred in the cocoanut treated series which also had the greatest depression at the start.

It will be noted that the increase in surface tension during the ten-day period is inversely proportional to the surface tension at the beginning of the experiment. In nearly all cases in the inoculated series the maximum increase in surface tension occurred within the first forty-eight hours. There was then in general a steady climb until the end of the experiment. The data show a tendency for the surface tension in the inoculated broths containing depressants to approximate that found in the uninoculated broth with no depressant, within seven to ten days. This shows that growth characteristics after several days' incubation cannot be attributed to a surface tension found at the onset of the experiment. This is brought out strikingly in chart 1.

Even in the absence of inoculation the surface tension of all broths containing depressants showed a decided increase within twenty-four hours. There was then some slight fluctuation throughout the period, but in no case did the surface tension drop to the level found at the onset, or increase to the point found in the control broth without depressant.

There was a steady increase in pH in all inoculated tubes until they reached 8.3 to 8.4 at the tenth day. Thus we find the surface tension and hydrogen ion concentration approaching a constant as the result of the metabolism of *Escherichia coli* regardless of the initial surface tension.

Growth of aerobes in broth of varying surface tension

The four soaps were added to stock broth in varying concentrations and hydrogen ion concentration and surface tension determined. Each experiment was so arranged that the pH of

all the tubes was approximately the same at the start. Tubes of each concentration were then inoculated with a twenty-four-hour culture of each of the following organisms: *B. subtilis*, *B. anthracis*, *Serratia marcesens*, *Pseudomonas aeruginosa*, *Eberthella typhi*, and *Staphylococcus aureus*. The tubes were then incubated at 28°C. and observations made after twenty-four and forty-eight hours and again at the end of one week.

The surface tension and soap additions are shown in table 2.

All the soaps except palmitic seemed to be somewhat toxic to both *B. subtilis* and *B. anthracis*. Palmitic proved to be stimulating to *B. subtilis* in forty-eight-hour incubation, and the organism produced a heavier pellicle with this soap at 33 dynes

TABLE 2

Surface tension of broth containing depressants

	CASTOR SOAP			OLIVE SOAP			PALMITIC SOAP			COCOANUT SOAP		
Cubic centimeter of 2 per cent solution per 100 cc. broth......	0.1	0.5	1.0	1.0	3.0	5.0	1.0	3.0	5.0	1.0	3.0	5.0
Surface tension, dynes per square centimeter.	43	35	33	33	32	31	37	34	33	44	38	30

Surface tension of control broth, 49 dynes per square centimeter.

than in the control of 49 dynes. At the end of a week this difference was not noted, the growth being about the same as that in the control. *B. anthracis* failed to develop well in any of the tubes containing soap.

Results were somewhat striking with *Pseudomonas aeruginosa* and *Serratia marcesens*, particularly the former. Both organisms showed stimulated pigment formation, heavier pellicles, and less growth in the body of the media, in all tubes which contained soap. Since castor oil soap was not toxic to *Pseudomonas aeruginosa* it was later employed in concentrations of 1, 3, and 5 per cent. These higher concentrations of the soap did not produce any further depression of the surface tension, yet the stimulation of pigment formation and pellicle, and diminution of growth throughout the body of the medium was more marked than in the previous concentrations. The observed stimulation

was then due to the nature of the depressant rather than to the lowered surface tension. This shows the importance of using several depressants in order not to ascribe observed phenomena to the surface tension rather than the nature of the depressant.

Eberthella typhi was apparently unaffected by any of the soaps, growth in the treated tubes differing in no way from the control. The soaps were toxic to *Staphylococcus aureus* in the higher concentrations, and no differences could be ascribed to surface tension.

These results do not agree with those of Larson who states that all pellicle formers cease to grow at the surface when the surface tension is below 45 dynes. The above experiments were repeated many times and in every case, especially with the two pigment formers, the pellicle formation was stimulated in the presence of the soap. This stimulation is not ascribed to surface tension. It must be borne in mind that the lowest surface tension obtained in the above experiment, 31 dynes per square centimeter, would be about 42 to 43 dynes as determined by the drop weight method.

Growth in Uschinsky's medium. The same organisms used in the above experiment were used in a similar experiment employing Uschinsky's medium instead of the stock broth. The surface tension varied from 26 to 71.6 dynes per square centimeter. Control flasks showed that the surface tension of the medium remained approximately the same after sterilization as before.

Pseudomonas aeurginosa and *Serratia marcesens* developed very intense pigments and grew well. The latter organism produced very little pigment in the medium containing cocoanut soap, but the pigment was intense in the other tubes. The differences noted could in no way be ascribed to the lowered surface tension.

Growth of anaerobes

Soaps were added to the stock broth containing 1 per cent glucose. The surface tension is shown in table 3.

The surface tension of the control broth was 53 dynes. Tubes of each concentration of each soap were then inoculated with the following anaerobes: *C. histolyticum*, *C. welchii*, *C. sporogenes*, *C. bifermentans*, *B. bellonensis*, *C. oedematis-maligni*, *C. oedematiens*, and two strains of *C. botulinum*. Controls consisted of an aerobic control, an anaerobic control, and a toxicity control for each organism. The aerobic control consisted of a tube of the glucose broth inoculated with the organism in the usual manner. The anaerobic control consisted of a tube of the glucose broth boiled for ten minutes, plunged in cold water, inoculated, and then sealed with a layer of sterile paraffin oil. The toxicity control consisted of tubes of the glucose broth containing the various soaps, boiled, and after cooling and inoculating, sealed with paraffin oil.

TABLE 3

Surface tenstion of glucose broth containing depressants

	CASTOR SOAP			OLIVE SOAP			PALMITIC SOAP		
Cubic centimeter of 2 per cent solution per 100 cc. broth	0.1	0.4	0.8	0.1	0.4	0.8	1.0	3 0	5.0
Surface tension, dynes per cubic centimeter	52	43	39	51	42	38	48	44	40

In no case did any of the anaerobes show any development in the tubes not sealed with paraffin oil. All showed excellent growth in the tubes treated with the paraffin seal. The toxicity controls in general gave good growth, some showing less growth than the controls. *C. botulinum* was completely inhibited by both palmitic and castor soaps, but grew well in the olive soap. This would indicate that lowered surface tension within the limits of this experiment, does not bring about the development of anaerobes under aerobic conditions.

Surface tension and nitrogen fixation by Azotobacter chroococcum

Ashby's medium received various concentrations of soap, as indicated in table 4, and was then inoculated with a soil sus-

TABLE 4

Surface tension and nitrogen fixation by Azotobacter chroococcum

NUMBER	TREATMENT	SURFACE TENSION PER CUBIC CENTIMETER	NITROGEN FIXED PER 100 CC.	
				Average
	per cent	*dynes*	*mgm.*	*mgm.*
1–2	Castor 0 1	54.5	9.1 9.3	9.2
3–4	Castor 0.5	43.0	8.3 9.1	8.67
5–6	Castor 1.0	40 3	11.8 10.4	11.1
7–8	Cocoanut 1.0	54.5	10.3 11.5	10.9
9–10	Cocoanut 3.0	40.3	10.1 12.0	11.05
11–12	Cocoanut 5 0	34 8	10 6 9.7	10.15
13–14	Palmitic 1.0	48 0	10.2 10.1	10.15
15–16	Palmitic 3.0	45.8	10.7 10.9	10.8
17–18	Palmitic 5 0	38 2	11.2 11.6	11.4
19–20	Olive 0.1	48.0	10.4 9.7	10.05
21–22	Olive 0.5	39 8	9.4 10.2	9.8
23–24	Olive 1.0	38.2	11.7 9.4	10 55
25–26	Controls	74.2	10.8 11 7	11.25

pension containing Azotobacter. The flasks were then incubated at 25°C. for two weeks and the total nitrogen determined.

The Azotobacter film was uniform, pigmented, and heavy on all flasks, little or no difference being noted. Table 4 shows the surface tension of the media and the amount of nitrogen fixed.

It is obvious that neither the lowered surface tension nor the presence of the depressants have materially affected nitrogen fixation by *Azotobacter chroococcum.*

Oil seals

Interesting results were obtained with various oil seals sometimes employed in the cultivation of anaerobes in broth. The surface tension of ordinary paraffin oil was found to be about 28 dynes, olive oil 34 dynes, and castor oil 35 dynes. By using these oils as seals over ordinary broth the difference in tension of the phases is lessened and consequently the tension is lowered. No change was noted in the development of an ordinary pellicle forming organism under such a seal, but *Eb. typhi* formed a well-developed pellicle at the two phases, particularly in the case of olive oil. This did not develop in the case of paraffin oil. Azotobacter developed a splendid film under a half-inch layer of paraffin oil, and fixed as much N. as in the control flasks. Other data with reference to interfacial tension and bacterial growth will be presented at a later date.

SUMMARY

The foregoing experiments show that one of the most important factors in work on the relation of surface tension to bacteriological activities is the nature of the depressants employed. Soaps were found to be very satisfactory depressants because a very small amount produced a material depression in the surface tension. There is a considerable difference in various soaps, not only in their ability to depress surface tension, but in the effect on the organisms. Error in observations can only be avoided by using several soaps in concentrations such that each produces similar depression in the surface tension. If an observed phenomenon does not occur with several depressants, at approximately the same surface tension, it cannot be cor-

rectly ascribed to surface tension, but must be due to the nature of the depressant. Keeping this in mind, and using four depressants, no case has been found in the preceding experiments in which variation from control might better be attributed to surface tension than to the influence of the depressants themselves.

No observation has been made which would lead to the conclusion that pellicle formation is suppressed at lowered surface tension. Organisms produced pellicles at a surface tension as low as 32 degrees per square centimeter as determined by the method of Fahrenwald. This would correspond to a surface tension of 42 to 44 dynes as determined by the drop weight method.

Anaerobic bacteria failed to develop under aerobic conditions even when the surface tension was greatly lowered.

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THE REDUCTION OF SELENIUM COMPOUNDS BY SPORULATING ANAEROBES

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One of the first duties of any author who refers to a living organism by its generic and specific names is to make sure by all means at his command of the identity of the form in question. This principle evidently was not followed by Levine (1925) in the case of a recent paper, containing a list of sixteen sporulating obligate anaerobes which he tested for their reduction of sodium selenite. These cultures were obtained from the collection formerly in the American Museum of Natural History in New York City, now maintained in Chicago, and were apparently accepted at their face value without any effort to check up their identity by criteria which are now well established. There is no practice so conducive to confusion or so detrimental to progress in the science of bacteriology as the willingness of workers to accept uncritically the names upon so called "authentic" stock cultures.

It happens that the writer (1922) studied ten of the cultures in question, not for their action upon selenium, but for their purity and identity. Only one of the ten, i.e., *B. sporogenes* 120, was pure and correctly named! "*B. welchii* 500" contained *B. welchii* mixed with *B. sporogenes;* "*B. welchii* 521" probably contained *B. welchii* but only *B. sporogenes* was isolated. "*B. tetani* 1" contained no pathogenic organisms but consisted of a highly putrefactive species that I have designated *B. tyrosinogenes* because of its marked liberation of tyrosin from protein media. This is the only strain of this species that has ever been recognized. "*B. tetani* 274" contained no tetanus bacillus but consisted of an unidentified anaerobe and *B. sporogenes.* The

remaining five cultures, though evidently pure, consisted of *B. sporogenes.*

The correspondence between the museum's designations and those of the writer, as published in 1922, is as follows:

Museum's designations	*Writer's designations*
B. welchii 521	*B. welchii* 2 contaminated with *B. B. sporogenes* 72
B. welchii 500	*B. welchii* present (?) contaminated with *B. sporogenes* 42.
B. welchii 20	*B. sporogenes* 114
B. feseri 48	*B. sporogenes* 122
B. tetani 274[1]	*B. sporogenes* 88, also unidentified anaerobe, not *B. tetani*
B. tetani 1	*B. tyrosinogenes* 106
B. sporogenes 120	*B. sporogenes* 84
B. oedematis 421	*B. sporogenes* 121
B. oedematis maligni 485	*B. sporogenes* 90
B. botulinus 595	*B. sporogenes* 44, 46 (?)

The actual serologic identity of the sporogenes strains was later shown by Hall and Stark (1923) by means of the agglutination test. The fact that all of the anaerobes tested by Levine reduced sodium selenite at 1:50,000 suffers considerable limitation when interpreted in the light of these facts. We may readily agree that *B. sporogenes* reduces sodium selenite but any conclusions regarding the other species of anaerobes mentioned must await more critical experiments.

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[1] This culture came to the writer indirectly from the museum through the Department of Bacteriology and Hygiene at the University of Chicago.

FURTHER OBSERVATIONS ON UTILIZATION OF THE SALTS OF ORGANIC ACIDS BY THE COLON-AEROGENES GROUP

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A previous study of the utilization of salts of organic acids by the colon-aerogenes group has shown that *Bacterium aerogenes* and its allies are capable of breaking down the sodium, potassium and ammonium salts of citric acid while *Bacterium coli* from the intestines of man and higher animals does not possess this property. Of the eighteen different organic acids employed, citric acid was the only one which permitted a clear cut distinction between these two types.

The present investigation represents a continuation of the previous work and in this instance attention has been centered upon several organic acids whose structure somewhat resembles that of citric acid. Information concerning the behaviour of the colon group toward these acids appeared desirable from several viewpoints. In the first place an opportunity was offered for studying the relationship of chemical constitution to bacterial utilization, and secondly, there was the possibility that structurally related acids might afford the same separation between the coli and aerogenes types and thus be of value as an additional means of separating the main sections of the group. In addition to citric acid, aconitic, tricarballylic, glutaric and adipic acids were employed. These were obtained from either American or British chemical houses or from the Chemistry Department of the University of Illinois.

In a recent study of the organic acids for differentiating various bacterial types, Brown, Duncan, and Henry (1924)

mention the employment of the sodium salts of aconitic, glutaric and citric acids in addition to many others. The organisms used in their study were chiefly members of the paratyphoid group though several colon-aerogenes cultures and others were included. They confirm the writer's observations on the value of sodium citrate for differentiating the colon group. They further state that the salts of aconitic and glutaric acids are apparently not readily decomposed by any member of the Salmonella group or allied types, though they do not mention the colon group specifically in this connection.

It should be noted that their method of testing utilization of the acids differed from that previously employed by the writer (Koser, 1923–1924). Instead of using a synthetic medium and supplying the acids as the sole source of energy, they added them to either nutrient broth or a pepton solution. Under these conditions it was found that gas production, a change in reaction, or a more luxuriant or retarded growth were not sufficiently reliable criteria of utilization. They then resorted to precipitation by means of lead acetate. This procedure afforded a reliable indication of utilization of many of the acids and a number of very interesting distinctions were brought out, chiefly between members of the paratyphoid group.

In the present study, the procedure for testing utilization of the acids was similar to that employed in former work (Koser, 1923). The basic medium consisted of 0.5 per cent sodium chloride, 0.02 per cent magnesium sulphate, 0.1 per cent dibasic potassium phosphate and 0.1 per cent ammonium acid phosphate and to this the various organic acids were added, one at a time, in amounts sufficient to give a concentration of 0.2 per cent. Each solution was then brought to pH 6.8 by the addition of sodium hydroxide solution so that the resulting media contained somewhat over 0.2 per cent of the sodium salt of each acid. Media were tubed in 5 to 6 cc. amounts and sterilized in the autoclave at 15 pounds extra pressure for fifteen to twenty minutes.

Representative cultures of each section of the colon-aerogenes group were tested in the foregoing media. The coli

section was represented by 40 cultures recently isolated from fecal material, the aerogenes section by 35 cultures obtained from soil. The soil strains had been isolated several years previously and kept in stock on agar slants for two to three years. Most of them failed to liquefy gelatin and thus would be classed as of the aerogenes rather than the cloacae type. Also, a number of other cultures of soil origin were used. These resembled the typical intestinal *Bacterium coli* in respect to the methyl red and Voges-Proskauer tests, but had been obtained from a different natural habitat and could easily be distinguished on the basis of the citrate test. In the following table they are designated the "intermediate type."

After inoculation of the different organic acid media, records of growth as determined by visible turbidity were made daily for the first four or five days and thereafter at more infrequent intervals up to thirty days. Determinations of the hydrogen ion concentration by the colorimetric method with the usual indicators were also frequently made as an aid in measuring utilization of the acids. The temperature of incubation was 30°C. throughout the work.

The results summarized in Table 1 represent development at the fourth day after inoculation. With certain exceptions which will be mentioned later, further incubation failed to bring out any striking changes from those observed at this time so that the record of results at the fourth day gives a fairly accurate picture of the development of the organisms in the different organic acid solutions. Citric acid was used for purposes of comparison in addition to the controls given at the foot of the table. The results secured with citric acid, employed as its sodium salt, are similar to those previously reported.

As shown in the first column of the table, *Bact. coli* was unable to make use of any of the acids with the possible exception of glutaric, though here the results are not conclusive since development was slight. Some of the aerogenes cultures and the intermediate type utilized tricarballylic and aconitic acids, though this ability was not possessed by all of them. Glutaric acid was utilized sparingly if at all, while adipic acid gave nega-

TABLE 1

Utilization of certain organic acids by the colon-aerogenes group

ORGANIC ACIDS EMPLOYED AS THE SODIUM SALT	BACT COLI; 40 STRAINS; METHYL RED + VOGES-PROSKAUER 0; HUMAN INTESTINE	INTERMEDIATE TYPE; 21 STRAINS; METHYL RED + VOGES-PROSKAUER 0; SOIL	AEROGENES SECTION; 35 STRAINS; METHYL RED 0, VOGES-PROSKAUER +; SOIL
Citric	(40) negative	(21) +++	(35) +++
Tricarballylic	(40) negative	(12) negative (9) +++	(29) negative (2) ? or + (4) +++
Aconitic, I	(38) negative (2) +	(18) ? or + (3) +++	(2) negative (22) ? or + (11) +++
Aconitic, II	(40) negative	(12) negative (6) ? or + (3) +++	(8) negative (16) ? or + (11) +++
Glutaric, I	(6) negative (34) ? or +	(6) negative (15) ? or +	(15) negative (20) ? or +
Glutaric, II	(12) negative (28) ? or +	(11) negative (10) ? or +	(26) negative (9) ? or +
Adipic	(40) negative	(21) negative	(31) negative (4) ?
Controls:			
1. Basic medium + 0.2 per cent glucose	(40) +++	(21) +++	(35) +++
2. Basic medium only, no added source of energy	(40) negative	(21) negative	(34) negative (1)* ? or +

Increasing luxuriance of growth from slight to heavy turbidity is indicated by the plus signs, +, ++, and +++. No evidence of growth is designated "negative." The numerals in brackets preceding these signs show the number of cultures in each case.

The observations given here are readings taken on the fourth day after inoculation. Incubation at 30°C.

* This one culture gave questionable or slight growth in the basic medium; upon several different tests, however, it failed to show growth upon successive cultures in this solution.

tive results. All of these acids failed to yield the clear cut distinction shown by citric acid.

A comparison of the structural formulae of the several acids is of interest in connection with the question of their utilization. In the following list succinic acid is included for purposes of comparison though it was not used in the present work.

Citric	Tricarballylic	Aconitic
$CH_2 \cdot COOH$	$CH_2 \cdot COOH$	$CH \cdot COOH$
$\vert$	$\vert$	$\Vert$
$C(OH) \cdot COOH$	$CH \cdot COOH$	$C \cdot COOH$
$\vert$	$\vert$	$\vert$
$CH_2 \cdot COOH$	$CH_2 \cdot COOH$	$CH_2 \cdot COOH$

Succinic	Glutaric	Adipic
$CH_2 \cdot COOH$	$CH_2 \cdot COOH$	$CH_2 \cdot COOH$
$\vert$	$\vert$	$\vert$
$CH_2 \cdot COOH$	CH_2	$(CH_2)_2$
	$\vert$	$\vert$
	$CH_2 \cdot COOH$	$CH_2 \cdot COOH$

It will be seen that the replacement of the hydroxyl group of citric acid by the hydrogen of tricarballylic renders the latter acid unavailable for many of the aerogenes and related cultures which readily attacked citric acid, while *Bacterium coli* refuses to grow in either case. In the dicarboxylic acid series represented by succinic, glutaric, and adipic acids, it was previously found (Koser, 1923) that succinic acid supports the growth of both *Bacterium coli* and *Bacterium aerogenes.* The higher members of this series seem to be less readily available, for glutaric acid permitted only a slight growth in some cases while adipic acid gave negative results. It is possible, also, that the results secured with glutaric acid may have been due to impurities and that this acid, like adipic, is not utilized.

The peculiar results given by glutaric acid and to some extent by aconitic acid deserve especial mention. Two samples of each of these acids were available for the tests and as somewhat different results were secured with each of the two different preparations, all are included in the table. In the first aconitic acid medium two strains of *Bacterium coli* developed

slightly, though all were negative in the medium prepared from the second sample. The intermediate type and the aerogenes cultures also gave somewhat different results in the two samples. In the first case only two strains of *Bacterium aerogenes* refused to grow, while 22 gave a questionable or slight growth and 11 produced a heavy vigorous growth with undoubted utilization of the acid. The questionable or slight growth was accompanied by little if any change in the hydrogen ion concentration, while the heavy growth always resulted in increased alkalinity. In the second sample eight of the aerogenes cultures were negative whereas six of these had shown questionable or slight growth before. The same eleven cultures which at first exhibited vigorous development again produced a heavy turbidity in the medium. A number of the "intermediate type" cultures have shown the same tendency toward questionable or slight growth in the presence of aconitic acid, though this was much more pronounced in the first sample.

Somewhat similar results were encountered with all of the cultures in the two samples of glutaric acid. In these cases the development was quite slow, a light turbidity appearing in from two to four days and in occasional cases not until the sixth or seventh day. A few aerogenes and several cultures of the intermediate group showed a somewhat heavier growth after two or three weeks, though this was accompanied by very little change in the hydrogen ion concentration.

In view of the foregoing results the purity of these two acids, especially the glutaric acid, may be questioned. The samples used were the best obtainable, but even though regarded as being chemically pure they may have contained traces of other compounds which affected the results in one way or another, either by supplying available foodstuffs and thus permitting some slight development or perhaps by retarding true utilization of the acid. There is some evidence to show that traces of impurities which can not be detected by the usual chemical or physical methods may be made evident by bacteriological procedures. Pfanstiehl and Black (1921) have pointed out the difficulty of determining the purity of sugars used in bacteriological work

since in many instances the chemical tests are insufficient. Kendall and Yoshida (1923) have shown that 0.0025 per cent of levulose in pepton solution may be detected readily by fermentation by *Bacterium coli* and that even the detection of 0.001 per cent was not impossible. Koser and Rettger (1919) found that the addition of 0.001 per cent of aspartic acid (neutralized) to a solution of inorganic salts and glycerol permitted slight development of a number of organisms. In a similar way Koser (1923) obtained growth by *Bacterium aerogenes* when 0.01 per cent sodium citrate was supplied as the source of energy, 0.001 per cent permitted some development though usually not enough for production of a visible turbidity.

The foregoing instances tend to show that the addition of very small quantities of available carbon- or nitrogen-containing compounds to a solution otherwise suitable for growth will be sufficient to bring about considerable multiplication. When submitting unknown compounds to bacteriological tests it follows that the determination of the melting point, to use but one example, may not be a sufficiently reliable criterion of purity and consequently results such as those secured with glutaric acid and in some cases with aconitic acid are open to several interpretations, since at present one can not be absolutely sure of the purity of the products tested.

It can be stated in a general way, however, that neither aconitic nor glutaric acids would be suitable for differentiation of the several sections of the colon group. Some strains of the aerogenes section fail to utilize aconitic acid and thus can not be distinguished from *Bacterium coli* by this means, while both the coli and aerogenes types exhibit about the same deportment toward glutaric acid.

SUMMARY

The utilization of several organic acids of the dicarboxylic and tricarboxylic series by *Bacterium coli*, *Bacterium aerogenes*, and related types has been studied.

Although several of these acids are structurally quite similar to citric acid, none of them afforded the same distinction be-

tween the intestinal *Bacterium coli* and other members of the colon-aerogenes group which may be brought out by means of citric acid.

Bacterium coli was unable to make use of any of the acids with the possible exception of glutaric and here the results were questionable. Some of the cultures of soil origin readily utilized tricarballylic and aconitic acids though others did not possess this property. Glutaric acid was utilized sparingly if at all while adipic acid gave negative results.

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THE PRODUCTION OF CATALASE BY AN ANAEROBIC ORGANISM

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The production of catalase by bacteria has been well known since the discovery of this enzyme, and the early recognition of its almost universal occurrence in living cells and tissues. Beyerinck (1893), however, long ago showed that it is not produced in demonstrable quantities by the lactic acid producing group of bacteria, and Orla-Jensen (1919) and others have followed Beyerinck in giving considerable weight to this characteristic in the differentiation of the true lactic organisms. In fact, McLeod and Gordon (1923) have suggested a classification of bacteria based upon catalase production together with the ability or inability to form hydrogen peroxide.

Recently catalase formation has taken on an especial significance in studies of the obligatory anaerobic bacteria, owing to the non-production of this enzyme by the well known anaerobes and the probable physiologic importance of this fact. According to McLeod (1925), Löwenstein (1903) was the first to note the absence of catalase in an anaerobe. Callow (1923) found all of the anaerobic cultures which she tested to be devoid of catalase. These observations on the absence of catalase production among the obligate anaerobes have been confirmed and extended by McLeod and Gordon (1923) and by Avery and Morgan (1924). Hagan (1924) has added the significant finding that a strict anaerobe belonging to an entirely different group from the commonly studied sporulating anaerobes, *Actinomyces necrophorus*, also lacks the ability to produce catalase.

The plausible hypothesis has been advanced that, since hydrogen peroxide is apparently not formed in significant quan-

tities under anaerobic conditions, the reason the obligate anaerobes do not grow in the presence of free oxygen is because of their inability to produce catalase to effect the destruction of the peroxide formed. Many relevant data have been recorded in support of this view; forming evidence so strong, in fact, that this interpretation appears to be the most logical explanation of the anaerobic natures of at least some members of this class of organisms. Such a view would clarify such facts as the apparent aerobic growth of anaerobes in the presence of plant or animal tissues, and their growth in contact with air when associated with certain aerobes.

Lest a general theory of anaerobiosis be constructed upon this foundation, it is perhaps pertinent to call attention to an apparent exception to the general rule. The group of propionic acid producing bacteria, at least the strains with which the present writer is acquainted, should be classed as strict anaerobes but these organisms are especially vigorous producers of catalase. Attention has been called to this marked characteristic of the propionic bacteria in connection with discussions of their significance in the Swiss cheese industry (Sherman (1919, 1921)).

The note here recorded conflicts in no way with the excellent researches on the correlation between anaerobic growth and inability to form catalase, nor with the interpretation which other workers have put on these facts. It indicates, however, that all obligate anaerobes are not devoid of catalase producing capacity, and that an hypothesis which seeks to explain anaerobiosis on that basis is probably not universally applicable.

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STUDIES IN MICROBIC HEREDITY

VII. OBSERVATIONS ON THE GENETIC ORIGIN OF THE SEVERAL TYPES OF FUNGI FOUND IN THE LESIONS OF BLASTOMYCOSIS HOMINIS

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From the standpoint of infectious etiology there exists a curious paradox, in regard to the organisms of blastomycosis. A disease, which is both clinically and pathologically an entity, appears to be caused by a group of organisms comprising at least four distinct types which vary in their morphology from a budding yeast form, to a mould type having aerial hyphae with fruit organs, and reproducing by endosporulation.

Obviously it is difficult to reconcile this state of affairs with our views of the specific infective capacity of single types of bacteria for the clinical entities that they produce. In order to conform to this view Ricketts (1901) regarded the several types as more closely related than their diverse morphology might indicate. Yet there are others who take the position that these types are really distinct species and therefore find difficulty in explaining their relationship to the disease.

On the ground that these organisms comprise a more or less homogeneous group as Ricketts believed, there would seem to be no good reason why more than one type should not be isolated from the same case of the disease. It is entirely conceivable that one might find a different type of organism in the young lesion than in the old, inasmuch as it is in the latter that they have been under the perpetual assault of the immunologic forces of the host. Such a situation would amount to a dissociation of types within the host tissue, in which event one should be able to isolate them.

In point of fact paper VI of this series (1926) has shown that this possibility has been realized in the case studied. In order to prove, however, that the various types belong to a common stock it is necessary that a pure line culture of one type—be transformed into one of the other types. It is partly the purpose of this paper to report our experiments on the transformation of the Type II yeast into the mould-like Type III yeast.

VARIABILITY OF TYPE II

In the previous paper on these fungi (1926) I outlined the general conditions under which ascospore formation took place

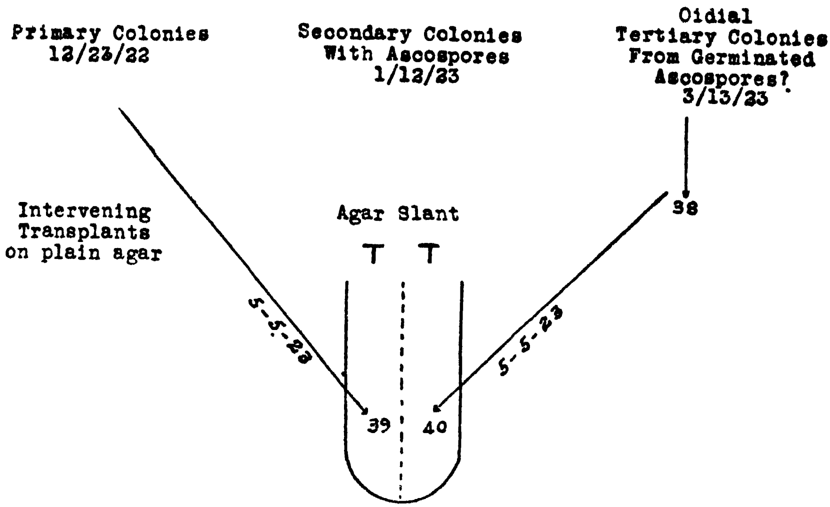

DIAGRAM 1.

and took occasion to refer to causative factors having to do with the strain itself which are not easily controlled.

With the aid of diagram 1 I shall now point my meaning by the elucidation of a most confusing paradox that we have observed on more than one occasion. This has to do with the fact that two strains of the same original pure line may later, when planted under identical conditions, yield life cycles of greatly varying degrees of complexity.

The Type II strain, transplant 33, was seeded on December 22, 1922, and developed on the dates indicated primary, secondary and tertiary colonies. Transplant 38 from the tertiary colonies preserved somewhat the larger oidial type of cell that characterized them. On May 5, 1923, as transplant 40 it was seeded on one side of the identical tube (*TT* in diagram) that received a seeding on its other side of the descendants of the primary colonies. The intervening transplants from the primary colonies were made on plain agar before the seeding of transplant 39 on May 5, 1923.

Transplant 40 grew rapidly and early formed a spreading mycelium. No. 39 grew slowly in discrete colonies with but slight tendency to form mycelium; yet there was morphologic identity in the cultures at this time. On June 15, 1923, no. 39 had developed a few insignificant secondary colonies in the upper or drying zone of the slant while no. 40 developed one large rough reddish secondary colony in the same zone.

On transplant this colony of no. 40 recapitulated the various steps in the anomalous method of reproduction shown in figures 16, 17, 18, 20 and 23 of paper VI of this series. On the contrary only the normal and oidial phases were seen in the secondary colonies of no. 39, and their transplants developed a cycle of relatively slight complexity. Yet the primary colonies of both nos. 39 and 40, as well as transplants from their secondary colonies, yielded the normal oval yeast phase of the organism, indicating the fundamental purity of both strains.

The essential difference between the two strains growing under exactly the same conditions involved the *range of their life cycles, and the origin of this difference appears to lie in the chromatic reconstructions of specialized cells* occurring in the secondary and tertiary colonies of transplant 33. The fact that these cells are of greatly increased viability is probably tributary to the major fact of their reorganization; indeed it seems possible that these chromatic changes require for their maturation a certain invulnerability of the cell.

Of great importance is the fact that the *descendants of these*

reorganized forms inherit some of the adaptability of which the forms themselves are the expression. This increased adaptability of their descendants is indicated by the readiness with which these phases of their life cycles are recapitulated. This expresses itself in secondary and at times in tertiary colonies. They signify that the pleomorphic cycle has been expanded to include special phases of development. Thus we have plausible explanation of the fact that two strains from the same original pure line, *planted under the same conditions*, may behave differently. The answer is simple: one strain has been rejuvenated through the characteristic reorganizations in the secondary colonies, but the other has not. One can only be assured of *keeping* hereditary lines pure if they are not permitted to pass through the sexual phase, or any other phase involving equivalent chromatic reorganization.

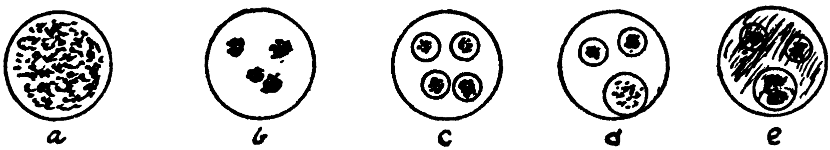

DIAGRAM 2.

Thus is explained in part *why* it may not be possible to formulate an environment that will permit the strains' expansion into all the *potential* phases of its cycle. If perchance the changes represented in diagram 1 occur in a strain without our observation, we are at a loss to explain the cyclic differences detailed above. The line is no longer pure even though the new biotype has characters that largely overlap those of the parent form. Had we been indifferent to the rôle of the secondary colonies we should have propagated the so-called normal phase only, and its lack of adaptability would have resulted in our loss of the strain. On the other hand, when the "normal phase" of any organism happens to be well adapted to ordinary media—as is indicated by its prolonged viability—the bacteriologist seldom sees the other phases, and when he does he calls them *involution* forms.

As further evidence for the microchemical reorganization that

such "involution" forms may undergo, are direct observations made on intravitally stained living forms.

The marked metachromasia shown by the nuclear material of these forms when stained in thionin arrested our attention. In many instances it stained a bright red, indicating a selective avidity for the polychrome component of the dye. This nuclear material is granular in form and may be located at either end of the cell or centrally (fig. 1 at *a*); some cells contained four of these nuclear collections. It is often sharply delineated from the rest of the cell and is surrounded by a clear space. The following changes were observed in cells staining diffusely blue.

The cell shown at *a* first became partially translucent as the result of concentration of the chromatin in bluish black granules (diagram 2 at *b*). These granules may at times migrate to the poles of the cell, or they may collect in its center. They were located in a large clear area and resembled somewhat a yeast nucleus (diagram 2 at *c*). The granules were actively motile and moved to all parts of the clear space.

In one of the larger cells containing four such collections two of them were seen to fuse much as drops of water run together (diagram 2 at *d*). Rather large bluish black granules broke up rapidly into a fine granular material, which in the twinkling of an eye became bright red in color. Within half an hour two rings of granules again appeared but the bright red color persisted (diagram 2 at *e*).

This changed chromatin at the end of the cells has been observed to initiate and actually to take part in the regular budding process of the cell (fig. 1 at *b*). Therefore it seems fair to conclude that this metachromatic reaction indicates a biochemical reconstruction on the part of the nuclear chromatin. It is probable, too, that it is of importance in the vital activities of the cell else it would not be concerned in reproduction. The rapidity with which this change from blue to bright red took place suggests physico-chemical alterations of a tauto-isomeric nature whose possible relation with chromatic reorganization I have detailed in a previous paper (Mellon, 1920).

THE BIOLOGIC ORIGIN OF THE MOULD TYPE NO. (III) FROM TYPE NO. II

We have attempted to indicate in our previous papers that with the bacteria the origin of new variants is associated with a type of chromatic reorganization similar to that shown in the secondary colonies of these fungi. Since the latter have yielded so many well recognized types of special growth forms it is not surprising that they have also proved the seat of origin for the mould-like stage of these fungi.

The poor viability of the normal forms of this strain has been an aid rather than a hindrance to the detection of the parent form of the new type. As nearly as can be pictured it is represented at *a* in figure 2. Normal sized forms are seen at *b*. The origin of this mould-like type has not been definitely traced to the zygospore—in fact we have seen no definite evidence of the latter process under the conditions of our observations.

The structures look like an oidium with a wall of great thickness. They recall the encysted oidium described by Guilliermond (1920). They closely resemble, too, certain chlamydospores, depicted in figure 25 of our previous paper (no. VI—1926) on these fungi. In any event they were the only forms in the culture capable of germination. This was proved not only by the fact that the normal forms looked dead and did not stain; it was shown also by the single cell technique, for regardless of how many normal cells were planted no growth occurred unless among them was one or more of the oidial-like structures just described.

The protocols detailing the origin of the variant are of special interest in connection with the expanded life cycle already described for transplant 33 (diagram 1). On January 24, 1923, a transplant was made from the ascospore containing secondary colonies of no. 33 to a partially dry agar slant to which 1 per cent maltose was added. The medium was a veal infusion, buffered to pH 6.0 with glycerophosphate and contained no pepton. The usual normal forms of the organism developed. The culture was sealed and kept at room temperature until September 10,

1923, when numerous fine black specks peppered the slant. In the absence of *sizable* secondary colonies these black specks, consisting of pigmented oidial forms, were not uncommon. Microscopically they appeared as the modified oidia referred to.

On transfer they rapidly grew into a spreading mycelium as seen in figure 3; they contrast sharply with Type II as seen in figure 4, in not yielding a creamy mass of buds starting from the center of the mycelial growth. From these mycelia spring long rows of oidial formation as seen in figure 5. Grossly, they appear as sharp tufts projecting from the surface of the culture.

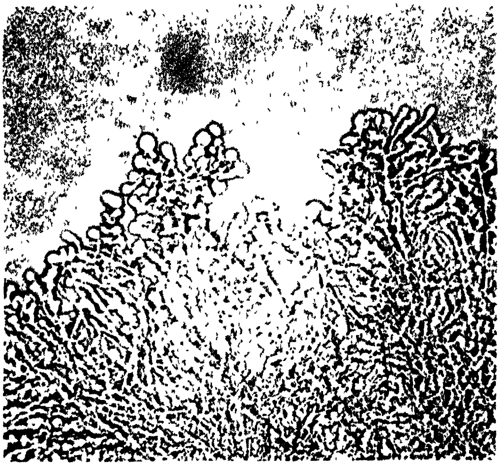

FIG. 5 THE VARIANT TYPE III SHOWING THE ORIGIN OF THE OIDIUM FROM THE MYCELIUM (FRESH UNSTAINED PREPARATION)

At this time "normal" germination was in abeyance. × 200

Definite aerial hyphae also form, but to date no fructification organs have developed from them.

For more than a year transplants of this culture reproduced only as the mycelial or the mycelial-oidial growth. Then buds began to originate from the mycelium identical morphologically with the normal forms of the maternal Type II. Secondary red colonies comprised of oidia indistinguishable from those of the black secondary colonies of Type II are not uncommon. When first dissociated the viability of Type III was fine, but

prolonged artificial cultivation has so decreased this that at present we are quite dependent on the red secondary colonies, unless we transplant at frequent intervals.

In the years that we have had this strain under close surveillance we have noted that this same variant has been reproduced six times. It has not always taken place on the same medium but the cultures from which it sprang have always been single cell ones. The change has been more or less spontaneous in that we did not start out to dissociate the variant. In fact many of these changes have come to notice by way of very close observation of what the organisms were doing *naturally*. Our attitude toward them has been not so much one of dictation as of "watchful waiting."

It is quite revealing what can be learned of microörganisms by merely transferring one's own cultures—not in the haphazard way of many modern technicians but with an eye always alert for the infinity of interest that living beings manifest. There can be no question that many beautiful anomalies are constantly discarded as contaminations by the unthinking technician; and to the extent that all of us cast aside from our daily routine the materials of perhaps valuable inquiry, we *are* technicians, regardless of our official status.

It was of interest that the antecedents of the Type III variant developed on a medium containing no pepton, but only germinated as the new form on a pepton-containing medium. Later the culture became adapted to the non-pepton media but without reverting to Type II. This illustrates a principle frequently repeated in our experience, and one that the mycologist understands better than the bacteriologist.

It can only mean that if we wish specialized stages to germinate we must frequently meet the organisms half way by providing an environment that will be compatible with the reorganizations which these special stages represent. On the other hand it must be appreciated that the reorganizations must be given a chance to ripen or mature, else they will not germinate on *any* medium. Only recently my Type III mould-like blastomyces failed to germinate, but a microscopical examination suggested

that immature oidia were developing from the mycelia. In about a month these blossomed forth as beautiful red secondary colonies and from them the original form of the Type III was then easily recovered. How easy it would have been to have discarded the culture as dead!

REACTIONS OF TYPE II AND TYPE III WITH SUGARS

Technique

Sugar free broth was prepared after Theobald Smith's method. To tubes containing 9 cc. of broth was added 1 cc. of 10 per cent

TABLE 1

TIME	GLUCOSE		LEVULOSE		GLYCEROL		LACTOSE		MALTOSE		MANNITOL		SALICIN		CONTROLS	
	Type II	Type III	Type II	Type III	Type II	Type III	Type II	Type III	Type II	Type III	Type II	Type III	Type II	Type III	Type II	Type III
days																
7	6 1	6 3	6 0	6 3	6.2	6 4	6 0	6 3	6 0	6.2	6.3	6.2	6.0	6.3	6.7	
	6 0	6 2	6 0	6.3	6 2	6 5	6 2	6 4	*6 0	6 2	6 3	6.3	6 2	6.4	6 8	
14	6 1	6 7	6 1	6 3	6.5	7 1	6 7	7.0	6 0	6.2	6 6	6.9	6.4	6.2	6.8	
	6.2	6 8	6 2	6 4	6 5	7 1	6 7	7.1	6 1	6.1	6.5	7.0	6 4	6 2	6 8	

* Methyl red used as control indicator.

solution of the various sugar solutions. They were grown for seven days at room temperature and titrated, and a duplicate set was titrated after fourteen days. The colorimetric method was employed. The results are in terms of pH. The indicators chiefly used were bromo-thymol blue and brom-cresol purple. Duplicate readings were made.

It is clear from table 1 that of the seven fermentable substances tested all were fermented with moderate acid production but no gas (one week). With lactose, Type II showed reversal of reaction at the end of two weeks, which was also partial for glycerol. Complete reversal or even alkali formation occurred with Type III in glucose, glycerol, lactose and mannitol. So although there is great similarity in the fermentative capacity of the

strains there is apparently some qualitative difference in the end products formed. The gross differences of the cultures are, to an extent, belied by the similarity of these tests which confirm their genetic lineage.

COMPLEMENT FIXATION RESULTS

The strains isolated from the patient were tested out serologically with his serum as well as with the sera of rabbits which had been inoculated with them. The agglutination results could not be regarded as satisfactory so fixation antigens were made of Types I and II. After a good growth had been obtained the organism was washed in salt solution and 0.1 per cent formol added. It was controlled for growth after four or five days and if sterile tested for its anti-complementary powers. If suitable, the antigenic dose of the antigen was determined by choosing one-fourth of the anti-complementary dose with normal serum. The antigenic unit in each case resulted in 0.1 cc.

When run against the patient's serum in 0.3 cc. a 4+ reaction with Type I antigen was obtained after thirty minutes incubation. A 3+ reaction was observed with Type II. After one hour, however, the reaction was only between 1+ and 2+. This delayed type of reaction and its probable significance I have already described in a previous communication (1921). Animals immunized against Type I organism produced a serum which fixed complement in 4+ in $\frac{1}{10,000}$ cc. in one rabbit and $\frac{1}{1000}$ cc. in another. A strain of *Endomyces albicans* (the thrush fungus) which had been isolated from an infected finger by Tanner gave a 4+ reaction in 0.1 cc. of the rabbit's sera but was negative in 0.01 cc. It is of interest that the heterologous Type II antigens showed greater fixing power for the serum of animals with Type I than did the Type I antigen. This occurrence is frequently paralleled, however, in agglutination work with bacteria.

DISCUSSION

As the result of our isolation of Types I and II blastomyces from a single case, coupled with the experimental transforma-

tion of Type II to Type III, Ricketts' original view of the close genetic relations of these types is confirmed. The law of Koch which predicates a morphologic type specificity for strains of organisms cannot be regarded as absolute. These morphologic types scarcely correspond to the *species* of the higher forms. They represent instead stages which have been stabilized or dissociated from the pleomorphic cycle of possibly a single entity.

Because the situation is strictly comparable to what our previous studies (1925–1926) have indicated for the bacteria the results have a peculiar interest. The parallelism with study no. III, which shows the true pleomorphism of *B. alkaligenes*, is especially noteworthy here because in its stabilized coccus, bacillus and filamentous branching types we have much the same complex as is presented by the morphology of the three types of blastomyces (endomyces). With the bacteria the complex is of a lower order of magnitude in the same sense that the solar system of the atom represents a lower order of magnitude than the solar system of the universe. In each instance the fundamental principle of arrangement is preserved.

There is another aspect of the situation that merits philosophic treatment since it has a really practical value in genetic work. I refer now to the difficulty of drawing a hard and fast line between environmental and intrinsic factors as they may determine variation within pure lines of organisms. Since it is practically impossibly to conceive of the existence of a living organism apart from an environment, I do not see how any one can limit variability changes *with certainty* to strictly intrinsic factors. Strictly speaking we cannot control environment since we are not even aware of all of its factors.

In our experience alterations in the hereditary mechanism occur in special stages of the organism, viz., in zygospores or their reorganization equivalents. The unfolding of these stages is conditioned by the environment at the time, *or by a previous environment.* So the behavior of an organism (in these respects) under a given environment could not be predicted short of an intimate knowedge of its full genetic history.

Eberson (1918) took the position that "different types, to be genuine mutants, must have developed when conditions remained constant." Aside from the great difficulty of knowing when environmental factors actually remain constant, this dictum is not consonant with the facts, as far as our experiments go. Neither does it conform with experiments of recent years in the field of experimental biology as I have previously pointed out (1920). There can be little question that a changing environment is of much significance in variational change, particularly when it is brought to bear on the germ plasm.

In confirmation of the isolation of more than one type (stabilized stage) of fungus from different lesions of the same patient we have the work of Rappaport and Kaplan reported at the (December, 1925) Meeting of the American Society of Bacteriologists. From a case of meningitis diagnosed antemortem by positive blood and spinal fluid cultures they obtained two types that parallel our Types I and II. They obtained them from different organs and have shown antigenic relation between them.

CONCLUSIONS

1. Following isolation of Types I and II yeasts from a single case of blastomycosis, the mould-like Type III has been derived genetically from pure line cultures of Type II.
2. The botanical mechanism for this transformation has been associated with specialized cells in the pleomorphic cycle of the organism. They resemble somewhat the chlamydospores.
3. The variation mechanism in general is the same as with the bacteria, although with the yeasts no definite evidence of true isoganic conjugation was observed in this instance.
4. The origin of very closely related biotypes also seems to be identified with the chromatic reorganizations of the pleomorphic cycle.
5. When conjugation or similar change occurs there can be no guarantee that the culture is *then* a pure line one.
6. The original view of Ricketts that the three distinct types really comprise a rather homogeneous group is sustained by actual experiment.

7. The isolation of Types I and II from lesions of different age makes it highly probable that variations occur in the host similar to those induced in vitro.

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EXPLANATION OF PLATE I

FIG. 1. METACHROMATIC REORGANIZATIONS OF NUCLEAR CHROMATIN IN GIANT YEAST CELLS AT *a*, WHICH AT *b* PARTICIPATES IN THE GERMINATION OF A NEW CELL

Intravitally stained in carbol-thionin. × 800

FIG. 2. REORGANIZATION FORMS OF SPECIAL RESISTANCE GROWING ON 2 PER CENT NaCl AGAR

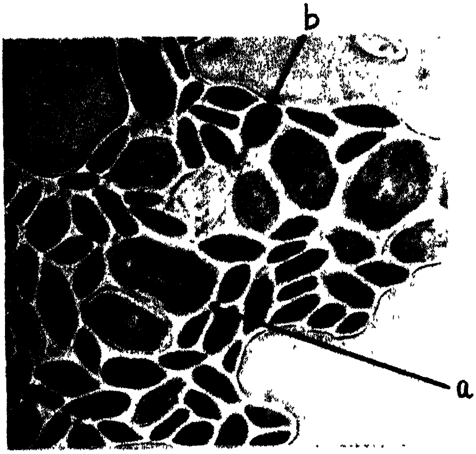
At *a* the parent cells for transition of Type II to the mould-like Type III. At *b* the poorly staining non-viable "normal forms." Carbol-thionin. × 800.

FIG. 3. THE MOULD-LIKE TYPE III DERIVED GENETICALLY FROM THE TYPE II YEAST

Note dominance of mycelial phase

FIG. 4. TYPE II

Note dominance of budding phase, which has overshadowed the scant mycelium which develops early.



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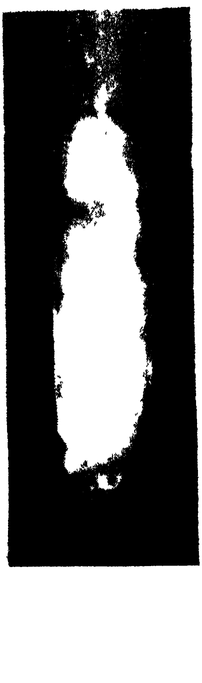

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4

(Mellon: Studies in microbic heredity)

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